- 運営指導調査団報告書 -

第１章　運営指導調査の概要

１－１　調査団派遣の経緯と目的

本調査は、「ペヘレイ増養殖研究開発計画」の終了にあたり、終了時評価調査団によって確認された残された技術的課題の現状把握を行うとともに、当該分野にかかる必要な助言・指導を行うことを目的とする。

なお、具体的業務内容は下記の通りである。

(1) C/P に対するマイクロサテライトにかかる技術移転の状況を確認するとともに、神奈川由来のペヘレイ親魚から得られる種苗の遺伝形質の解明を行う。(遺伝形質解析分野)

(2) 環境要因（水温、日照時間、給餌量）がペヘレイの繁殖活動に及ぼす影響にかかる研究の状況を確認するとともに、当該分野 C/P の指導を行う。(催熟技術研究開発分野)

１－２　調査団の構成と調査期間

１－２－１　調査団員

ストルスマン・カルロス・アウグスト(催熟技術研究開発分野)　東京海洋大学 海洋科学部 助教授

坂本 崇（遺伝形質解析分野）　東京海洋大学 海洋科学部 助教授

１－２－２　調査期間

2005 年 8 月 10 日～2005 年 8 月 24 日

月	日	曜日	行　　程	宿泊地
8	10	水	成田（19:00）→	機中泊
	11	木	サンパウロ(06:10)　RG8837 サンパウロ(07:30) → ブエノスアイレス(10:10)　RG8640 JICA アルゼンチン事務所打ち合わせ ブエノスアイレス → チャスコムス(陸路移動)	チャスコムス(アルゼンチン)
	12	金	(催熟技術)　INTECH・EHC にて活動内容打ち合わせ (遺伝形質)　INTECH にて活動内容打ち合わせ，実験設備点検	同上
	13	土	(催熟技術)　INTECH にて性別と繁殖活動の指標および産卵誘発に関する実験・技術指導・確認 (遺伝形質)　資料整理、遺伝形質解析にかかる実験・技術指導・確認 変性ポリアクリルアミドゲル作成	同上
	14	日	(催熟技術)　資料整理、INTECH・EHC にて活動内容打ち合わせ (遺伝形質)　遺伝形質解析にかかる実験・技術指導・確認 Primer の RI 標識、RI 標識 Primer を用いた PCR，電気泳動サンプル調整，変性ポリアクリルアミドゲル電気泳動，変性ポリアクリルアミドゲル乾燥，変性ポリアクリルアミドゲルの X ray film 感光	同上
	15	月	(催熟技術)　EHC にて産卵活動の観察、受精率・卵質評価に関する指導 (遺伝形質)　資料整理	同上
	16	火	(催熟技術)　EHC にて性別と繁殖活動の指標に関する実験・技術指導・確認 (遺伝形質)　遺伝形質解析にかかる技術指導 遺伝情報解析結果のデータ収集	同上
	17	水	(催熟技術)　EHC にて受精過程・精子運動性に及ぼす塩分の影響の調査 (遺伝形質)　遺伝形質解析にかかる技術指導 遺伝情報解析結果のデータ収集、まとめ 遺伝情報解析ソフトの説明	同上
	18	木	(催熟技術)　INTECH にて産卵誘発に関する実験・技術指導・確認、EHC にて産卵活動の観察、受精率・卵質評価に関する指導 (遺伝形質)　遺伝形質解析にかかる実験・技術指導・確認 X ray film 現像、遺伝情報解析結果判定評価、 Primer の RI 標識、RI 標識 Primer を用いた PCR、電気泳動サンプル調整，変性ポリアクリルアミドゲル電気泳動，変性ポリアクリルアミドゲル乾燥、変性ポリアクリルアミドゲルの X ray film 感光	同上

	19	金	(催熟技術)　EHC にて性別と繁殖活動の指標に関する実験・技術指導・確認、INTECH にて種苗の標識（高低焼印）に関する実験 (遺伝形質)　遺伝形質解析にかかる技術指導、 遺伝情報解析ソフトを使用したデータ解析	同上
	20	土	(催熟技術)　INTECH にて性別と繁殖活動の指標および産卵誘発に関する実験・技術指導・確認、種苗の標識（エラストーマ）に関する実験 (遺伝形質)　資料整理、遺伝形質解析にかかる技術指導、 遺伝情報解析ソフトを使用したデータ解析	同上
	21	日	(催熟技術)　資料整理、今後のプロジェクト検討課題打ち合わせ (遺伝形質)　遺伝形質解析にかかる実験・技術指導・確認 X ray film 現像、遺伝情報解析結果判定評価、 遺伝情報解析ソフトを使用したデータ解析、 今後のプロジェクト検討課題打合せ	同上
	22	月	チャスコムス → ブエノスアイレス(陸路移動) JICA 事務所への報告 ブエノスアイレス (20:20) →	機中泊
	23	火	トロント (06:35)　AC 095 トロント (13:25) →	機中泊
	24	水	成田 (15:50)　AC 001	―

第２章　調査団活動内容と成果達成状況

２－１　活動内容

（１）遺伝形質解析分野

＜これまでの経過と調査団到着時の現状＞

2003 年 11 月に、今回の調査団員でもある坂本団員が、INTECH に短期専門家として派遣され、分子遺伝マーカー(Microsatellite marker）を用いた遺伝情報の収集技法の指導を行った。この際、実験に用いた電気泳動槽と電源供給機材は、他の研究室の物品を借用した。この短期専門家派遣により、亜国研究者の遺伝情報の収集技法についての理解、技術伝達がなされたが、電源供給機材の出力不足が明らかとなり、新規機材の購入を提案した。一方、電気泳動槽に関しては、坂本団員が日本で使っている機材と基本的に同一構造のものであり、問題がないと判断した。

また、2003 年 12 月から約３ヶ月間、INTECH の Mr. Gaston Guilger が坂本団員の研究室において研修を受け、実際に本プロジェクトのサンプルについて遺伝情報の収集を行い、データ収集で成果を挙げ、十分な実験技術は習得したと判断した。

今回調査団到着時の現状としては、新規購入機材を用いた INTECH での遺伝情報の収集において、安定したデータ収集ができていない状況と判断された。その問題点としては、不慣れな新規購入機材（電気泳動槽と電源供給機材）の使用によるものと考えられたが、電源供給機材とともに、特に電気泳動槽はこれまでの研修で用いていた機材と異なる構造であり、そこにも問題点があると考えられた。2003 年 11 月に坂本団員が用いた電気泳動槽は、研究者の移動により借用が困難となり、新たに電気泳動槽の購入をしたとのことであったが、新しい実験機材を用いた安定的な実験系の構築は、短期の研修では習得が難しい技術力のため、新規電気泳動槽の購入の際に、適切なアドバイスができなかった点が惜しまれた。

＜活動内容＞

① 分子遺伝マーカー(Microsatellite marker）を用いた遺伝情報の収集技法の確認、指導。
② 新規購入機材を用いた実験系の構築、実験系の最適化。
③ これまでに得られている遺伝情報の解析ソフトを使用したデータ解析指導。
④ 亜国で生産されたペヘレイ種苗の評価。

（２）催熟技術研究開発分野

＜活動内容＞

① 外部指標による性別の判定および繁殖活動の推定ならびにバイオプシーを用いた卵巣の成熟度の調査に関する技術指導。
② 環境要因、特に水温と日照時間がペヘレイの繁殖活動に及ぼす影響の解明と産卵誘発技術開発。
③ 受精・発生過程・卵質評価に関する技術指導ならびに受精過程に及ぼす塩分の影響の調査。
④ 種苗の標識方法の検討。

２－２　達成状況

（１）遺伝形質解析分野

① 分子遺伝マーカー(Microsatellite marker）を用いた遺伝情報の収集技法の確認、指導。

安定的なデータ収集ができていないことから、使用している試薬類の調整ミス等を考え、試薬類の再調整、異なる実験条件の検討などを行った。試薬類、実験条件などには特に問題点はなく、Mr. Gaston Guilger の日本での研修実績もあり、細かい注意点はあったが、実験技術としては十分に習得していると判断した。

② 新規購入機材を用いた実験系の構築、実験系の最適化。

これまでに行なってきた通常の遺伝情報収集技法においては、電気泳動は定電圧（約 1500V から 1800V）で行なってきた。この常法では、変性ポリアクリルアミドゲルは 50 度程度に暖まり、泳動時間も 1.5～２時間程度で終了する予定である。新規購入した機材では、約 1500V から 1800V の定電圧では変性ポリアクリルアミドゲルはほとんど暖まらず、さらに泳動時間も５～６時間を要するなど、問題点が明らかとなった。そのため、3000V の定電圧での電気泳動を行ったところ、泳動時間は約２時間程度になったが、変性ポリアクリルアミドゲルが 50 度以上に暖まったため、電圧調整が煩雑となった。またゲルが乾燥したためひび割れが生じ、解読不明になるなど新たな問題点が生じた。ゲルの乾燥は、高温に熱せられたゲルをガラス板から

分離する際に急激な乾燥が起こることを明らかになったため、電気泳動後にガラス板を水冷し、室温程度に下がったところでゲルを分離することで対応できることがわかった。また、3000V もの定電圧で電気泳動することに関しては、その理由は不明であるが、INTECH が新規購入した電源供給機材の初期不良問題である可能性もある。新規購入した電源供給機材が米国製（米国内 120V）であり、亜国内 220V であることから、なんらかの問題が生じている可能性がある。電圧調整が煩雑な点は、定電力（1200W）で電気泳動することで、改善され実験可能であることがわかった。また、電気泳動像の結果がやや不明瞭になっている点に関しては、ゲル板ガラス表面の汚れが原因であると考えた。しかし、日本での研修で用いた電気泳動槽では、泳動槽とゲル板ガラスが分離できる構造のものを使用していたが、新規に購入された電気泳動槽は、泳動槽とゲル板ガラスが一体となっている構造で、ゲル板ガラスに泳動槽のプラスチックが結合してしまっているために、希塩酸による汚れ除去が困難な状態にあった。滞在中には汚れの除去を実施することができなかったが、プラスチック部の腐食を避けて酸もしくはアルカリ溶液での実施方法を指導した。

③ これまでに得られている遺伝情報の解析ソフトを使用したデータ解析指導。

今回の解析で用いている分子遺伝マーカーである Microsatellite marker は、集団の微細な違いを検出可能な非常に高感度な解析ツールである。

調査団が到着時に収集されている遺伝情報データには、新規機材への適応問題から、未完成の部分があり、実際に解析に使用できるデータは、Mr. Gaston Guilger が日本において収集したデータの２つのデータセット(５集団；Chascomus 湖産、Kanagawa 県産、Junin 湖産、 Salada Grande 湖産、Chasico 湖産、１マーカー；Obo01TUF）と(２集団：Chascomus 湖産、Kanagawa 県産、２マーカー；Obo01TUF、 Obo02TUF）の遺伝情報結果のみであった。１集団には約 50 個体を使用している。

それぞれの集団の違いを解析するためには通常３～５マーカーを用いることが一般的であり、そのため今回の解析では不十分であると考えられた。

１つ目のデータセットでは、解析ソフト Genepop(http://wbiomed.curtin.edu.au/genepop/）を用い、２つ目のデータセットでは、解析ソフト Arlequin(http://lgb.unige.ch/arlequin/）または Genepop を用いて解析した。１つ目のデータセットの解析結果としては、５集団全ての組み合わせにおいて集団の違いが検出された。また、２つ目のデータセットの解析結果では、Obo01TUF と Obo02TUF の２マーカーを用いた解析で２２集団に違いが検出された。Obo01TUF と Obo02TUF のそれぞれ１マーカーでの解析では、Obo01TUF のみを用いた解析では２集団に違いが検出され、Obo02TUF のみを用いた解析では２集団に違いが検出されなかった。１つ目のデータセットでも用いている Obo01TUF は、集団の微細な差を検出する高感度な分子遺伝マーカーであると考えられた。これらの解析ソフトの使用方法等について説明し、解析方法の理解、技術伝達がなされたと考えられた。

④ 亜国で生産されたペヘレイ種苗の評価。

③の解析で各集団間の違いが検出されたが、１つ目のデータセット（５集団、Obo01TUF）を用いて各集団間の遺伝的分化度の大きさを示す F_{ST} 値を求めた。(F_{ST} 値が 0 に近いほど２集団間の分化度が小さい。）

表　Obo01TUF マーカーによる各集団間の F_{ST} 値

	Chascomus	Kanagawa	Junin	S.Grande
Kanagawa	0.0637			
Junin	0.1062	0.2521		
S.Grande	0.0822	0.0842	0.2658	
Chasico	0.0479	0.1825	0.0236	0.1779

この結果を見ると、Chascomus 湖産のペヘレイは、Chasico 湖産(0.0479)、Kanagawa 県産(0.0637)、Salada Grande 湖産(0.0822)、Junin 湖産(0.1062)の順に F_{ST} 値が小さいことがわかる。また、Chasico 湖産と Junin 湖産の F_{ST} 値が 0.0236 と小さな値となった。

サンプリング地点が地理的に離れていること、水系が異なることなどから、 Chascomus 湖産と Chasico 湖産間、Salada Grande 湖産間、および Chasico 湖産と Junin 湖産間の F_{ST} 値に疑問を持ち、調査団のストルスマン・カルロス・アウグスト博士、 Dr. Gustavo Manuel Somoza、Lic. Gustavo Berasain と議論したところ、

Chascomus 湖には以前に Salada Grande 湖産のペヘレイが放流されていた事実と、本解析に用いた Chascomus 湖産サンプルを捕獲した年に Chasico 湖産のペヘレイが放流されていた事実を確認した。さらに、 近年に Junin 湖には Chasico 湖産のペヘレイが放流されていた事実を確認した。わずか１つの分子遺伝マーカーを用いた解析ではあるが、近年の亜国の放流事業と連動する結果を得た。亜国では、その年々で資源量な豊富な湖のペヘレイを用いて種苗放流がなされてきた経緯があり、現在の集団構造が以前からの集団構造を反映していないと考えることができる。

ところが、Kanagawa 県産のペヘレイはこの放流事業が展開される以前の Chascomus 湖産ペヘレイに由来しており、資源が豊富であった 50 年以上前の Chascomus 湖の集団を反映している可能性もある。よって Kanagawa 県産の集団は、今後の放流事業にとっても非常に貴重な集団であるといえる。これまでの亜国の Chascomus 湖への放流事業経過では、すでに Chascomus 湖を含む多くの湖に、集団の異なる Salada Grande 湖産、Chasico 湖産、Junin 湖産等を放流しており、今回解析した Kanagawa 県産および Chascomus 湖産のペヘレイは、Chasico 湖産と Junin 湖産の集団と、Salada Grande 湖産の集団の中間的な集団であると考えられた。

（2）催熟技術研究開発分野

① 外部指標による性別の判定および繁殖活動の推定ならびにバイオプシーを用いた卵巣の成熟度の調査に関する技術指導。

ペヘレイの催熟技術開発の一環として、まず、INTECH および EHC の C/P に外部指標による性別の確認および繁殖活動の推定方法を指導した。とりわけ、実習形式でペヘレイの解剖学的特徴（肛門、生殖孔、泌尿孔、泌尿生殖孔）および生殖腺の構造、ならびに外部特徴と生殖腺の発達度合との関連性について説明した。この知見を基に、雌雄の判別は確実になるほか、親魚選別に際して比較的簡単に産卵可能な個体を見出すことができる。さらに、バイオプシー(カニューレション）方法により卵巣の一部を採集して、非破壊的にかつ正確に卵巣の発達度合いを調べることができる。この方法を C/P に指導すると同時に、INTECH および EHC の親魚候補の成熟度を調べた。その結果、EHC では卵巣がよく発達しており、産卵開始直前の状態であると判断したのに対して、INTECH の親魚の大半は繁殖活動が停止していることがわかった。その原因は現時点で解明されていないが、栄養状態や水質（ガス病が発生している）などの可能性を提示し、今後に C/P が行う追加検討により明らかにする予定。なお、詳細および提言は英文レポートに記載されている通りである。

② 環境要因、特に水温と日照時間がペヘレイの繁殖活動に及ぼす影響の解明と産卵誘発技術開発。

ペヘレイは直径約 1.6㎜（1.4~1.8）で比較的大型の卵を産む反面、繁殖活動がピークである２～３年魚では１回の産卵で５千粒程度しか産まない。また、ペヘレイは多回産卵型で、１産卵期に数回にわたって産卵するが、個体間で繁殖活動が同調していないため短期間で大量の受精卵を確保することが困難である。そのため、種苗生産効率を向上させ、計画的かつ集約的に良質卵を確保するために、環境条件（水温・日照）の操作による産卵誘発技術が必要と判断された。亜国における産卵誘発技術を確立するために、INTECH の C/P に対して技術指導し、ペヘレイの繁殖活動に及ぼす日照条件の影響を解明するための二つの実験を開始した。なお、本実験は時間がかかるため、専門家が帰国後もアルゼンチン側が主体となって継続している。なお、詳細および提言は英文レポートに記載されている通りである。

③ 受精・発生過程・卵質評価に関する技術指導ならびに受精過程に及ぼす塩分の影響の調査。

卵質に影響を及ぼす要因（親魚の栄養状態や飼育条件など）を改善することを最終目的として、卵質の評価について指導を行った。その過程において、卵成熟過程の説明と排卵に至るまでの時間の推定、完熟卵と過熟卵の見分け方や受精卵と未受精卵の見分け方について指導した。一方、INTECH と EHC で、これまでに自然産卵によって得られた卵の受精率は概ね 30～50%である。それに関して、Chasico 湖でペヘレイは比較的塩分濃度の低い支流で産卵することや高濃度の NaCl がペヘレイ精子の運動を抑制するとの報告がなされている。また、塩分濃度が 15 ppt である INTECH において特に受精率が低いことから、受精に対する塩分濃度の影響を明らかにする必要があった。そこで、塩分濃度 0、5、10 と 15 ppt において胚盤と囲卵腔の形成や表層反応の有無、卵膜の透明度ならびに精子の運動性と受精率を調べた。その結果、塩分濃度 15 ppt までは特に受精に対する悪影響は認められなかった。なお、詳細および提言は英文レポートに記載されている通りである。

④ 種苗の標識方法の検討。

種苗放流における天然個体と人工種苗を区別するために、種苗の標識が必要不可欠である。そこで、孵化場にて容易に種苗を標識し、なおかつ標識再捕法によって放流効果を推定するためには、なるべく概観から標識の存在が確認できることが望ましいことから、体重 3～27g のペヘレイを用いてはんだごてによる皮膚の高温焼印、液体窒素による皮膚の低温焼印および胸鰭基部における蛍光エラストーマのインプラントによる標識の有効性を検討した。その結果、三つの方法とも特に難しいことではなく、一個体に付き 10～30 秒程度で容易に標識が可能であることがわかった。また、標識から一ヶ月たったところで、特に生残・成長に対する悪影響が見られない。今後はアルゼンチン側が主体となり、さらに観察を続けることで、成魚までに標識が検出できるかを調べる予定である。なお、詳細および提言は英文レポートに記載されている通りである。

２－３　プロジェクト事業進捗に果たした調査団業務の役割

(１）遺伝形質解析分野

これまでに収集されたデータは、２つのデータセット(５集団；Chascomus 湖産、Kanagawa 県産、Junin 湖産、Salada Grande 湖産、Chasico 湖産、１マーカー；Obo01TUF）と(２集団：Chascomus 湖産、Kanagawa 県産、２マーカー；Obo01TUF、 Obo02TUF）と不足しているが、その中で、本プロジェクトで生産された種苗の評価を行うことができた。さらに近年の亜国のペヘレイ放流事業と連動する結果を得られ、議論できたことは、とても重要な進展であったと考えられる。今回の遺伝情報解析技術は、今後生産される放流種苗の遺伝的多様性の保全・調査にも有効な方法であることも評価できる。また、新規購入機材による問題点も改善でき、今後、安定的なデータの収集が可能になると期待される。

（２）催熟技術研究開発分野

亜国ペヘレイ増養殖計画は、試験的種苗生産から大量生産へ、また本格的な放流事業へ移行する段階に至っており、これまでに問題でなかった課題が見えてきた。とりわけ、計画採卵や集約採卵、特に産卵誘発による短時間での大量の良質卵の確保が必要となっている。今回の派遣で C/P へ基本的な技術を指導したことにより、今後は現地における繰り返し実験からデータを収集・解析することで、効率的な採卵技術の開発へつながると期待できる。さらに、標識放流のための新しい標識方法の検討が行われたことから、今後はこの技術を活用して放流後の種苗の追跡や標識再捕法による放流効果の推定と放流方法（時期と場所、種苗の大きさなど）の改善が可能になると思われる。

２－４　今後の課題

(１）遺伝形質解析分野

これまでのアルゼンチンにおけるペヘレイ放流事業は、地域の集団構造を考えることなく進められてきたことから、今後アルゼンチン側が主体となり、本格的なペヘレイの資源保全・修復を考える際には、これらのことが問題となることも予想される。そのため、資源が豊富であった 50 年以上前の Chascomus 湖の集団がどのような集団であったのか(Kanagawa 県産に近い集団であると予想される）を調べることも、今後の重要な課題であると思われる。このことは、かなり困難な課題ではあるが、博物館などに保存されている古い標本を用いた解析や、過去にペヘレイが存在していなかった湖へ Chascomus 湖の集団が放流され、それ以後に他の放流がされていない湖でのサンプルの解析も有効であると考えられる。

（２）催熟技術研究開発分野

ペヘレイの産卵生態や繁殖生理、栄養要求などの基礎的な生物学知見に不明な点が多く、今後はアルゼンチン側が主体となってこれらの課題に取り組むべきと思われる。さらに、今後の資源増殖に向けた展開を考えた場合には、放流効果を推定するための方法論の整理や増養殖の普及員の要請、ならびに水系と水産資源の利用や漁業に関する法律の整理と整備が急務と思われる。

別添資料１：主要面談者

主要面談者

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・ 佐藤 功
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別添資料２：催熟技術研究開発分野英文レポート

Outline of activities and recommendations from evaluation mission on the JICA Project of Pejerrey Propagation and Aquaculture in Argentina (August 11th-22nd, 2005)

Dr. Carlos Augusto Strüssmann
Associate Professor
Tokyo University of Marine Science and Technology

Field of expertise:

Reproduction control, seed production and restocking

Field of activities:

Development of methods for mass-, synchronized spawning of high quality eggs and marking of seeds for release

Institutions visited:

Instituto Tecnológico de Chascomús (INTECH)
Estación Hidrobiológica de Chascomús (EHC)

Contents:

1- Outline of activities

1.1- Evaluation of external indicators of sex (maleness/femaleness) and of reproductive condition

Purpose: To clarify criteria to distinguish males and females from external body features such as the anatomy, size and color of the genital pore as well as to use these features to estimate the approximate gonadal condition of females with minimum stress. This activity was conducted both at the INTECH and the EHC.

Materials and Methods: Broodstock from the INTECH (August 13th and 20th, 2005; 4-year-old fish from the Kanagawa strain) and EHC (August 16th and 19th, 2005; 1.5-year-old fish from the Kanagawa strain) were used. A summary of the information on these fish and on other broodstock currently kept at the two institutions is shown in Table 1. Animals were briefly anesthetized in 100 ppm benzocaine prior to the observations.

Table 1. Broodstock maintained at the INTECH and EHC (as of August 31st, 2005)

Institution	Code	Tank (pond) type and size (ton or m^2)	Strain	Age (years) and generation	Number and sex ratio (♀:♂)	Total length (cm) (mean±SD)	Body weight (g) (mean±SD)
INTECH	KA	Canvas (20 ton)	Kanagawa	4 (1)	240 (1:1)	35.7±3.2	405.6±70.5
	KB	Canvas (20 ton)	Kanagawa	4 (1)	246 (1:1)	35.6±0.6	359.0±20.7
	K1	Canvas (20 ton)	Kanagawa	1.5 (2)	458 (1:1)	31.5±0.4	244±8.9
	Junin	Canvas (20 ton)	Junin	4 (1)	220 (1:1)	28.1±0.3	180.1±8.1
EHC	A3	Canvas (20 ton)	Kanagawa	1.5 (2)	400 (1:1)	-	-
	B2	Canvas (100 ton)	Kanagawa	1.5 (2)	1600 (3:1)	-	-
	A1	Canvas (20 ton)	Kanagawa	1.5 (2)	400 (1:1)	-	-
	Junin	Pond (100 m^2)	Junin	4 (1)	-	-	-

Results and Conclusions: As reported previously by workers from the Kanagawa Prefecture Fisheries Experimental Station, females generally have three openings (the anus, genital pore, and urinary pore, in this order from head to tail) in the pubic area. Males, on the other hand, generally have two (the anus and the urogenital pore, whereby the vas deferens and the urethra fuse beneath

the surface and appear superficially as one single pore). This inspection must be done carefully and with consideration to multiple features, however, because of the exceptional appearance of females with two and males with three openings. Thus, it is advisable to press slightly the abdomen to cause the enlargement of the pelvic area, so as to improve the visibility of the openings (Figs. 1, 2).

The genital pore of females appears either as a as a relatively wide, deep opening transparent at the surface but pink to gray underneath (Fig. 1), a barely visible transversal slit between the anus and the urinary pore (Fig. 2), or as a protruding papilla. The last condition is usually associated with imminent ovulation and spawning, but may also indicate the extraordinary retention of ovulated oocytes in the ovary and overripening. There is no clear-cut association of the former two conditions with reproductive status. However, the former is often found in senile females whereas the second appears commonly in "resting" or early vitellogenic females. Males have a relatively tight urogenital pore that already is or becomes reddish upon slight pressure around the pore due to the rich blood irrigation in the surrounding tissues. Frequently it appears as a red ring with a gray or white (due to the presence of sperm) narrow center (Fig. 3). The width of the white central (longitudinal) band in the belly of pejerrey is sometimes considered to be a distinctive feature between males and females. However, with the exception of the case of ovulating females, when the band becomes very wide, this criteria alone is not a reliable indicator of sex whereby there is frequent interpretation error caused by differences in the nutritional status of the individuals.

1.2- Assessment of the reproductive condition of the broodstock

Purpose: To estimate the degree of gonadal development and the possibility of spawning during the current reproductive season of the broodstock kept at the INTECH and the EHC.

Materials and Methods: Broodstock in the INTECH (August 13th-20th, 2005; 4-year-old fish from the Kanagawa strain) and EHC (August 16th-19th, 2005; 1.5-year-old fish from the Kanagawa strain) were harvested, anesthetized as described previously, and checked for reproductive conditions by observation of the appearance of the belly and the (uro)genital pore, as indicated in 1.1 and in some females also by biopsy (cannulation; Figs. 4, 5, and 6) of the ovary. Males were gently squeezed in the abdomen to verify the presence of collectable milt. In another two stocks from the INTECH (1.5- and 4-year-old fish from the Kanagawa strain Junin strains, respectively) inspection was performed only by careful observation of the fish without removal from the tanks, in order to minimize stress.

Fig.1 Appearance of the pelvic region of females

Fig.2 Appearance of the pelvic region of females

Fig.3 Appearance of the pelvic region of males

Fig.4 Cannulation of females at the INTECH

Fig.5 Cannulation of females at the EHC

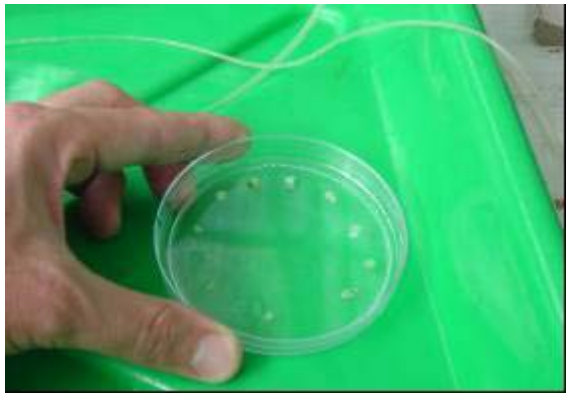

Fig.6 Cannulation of females at the INTECH

Results and Conclusions: Observation of the degree of ovarian development in females and the presence of collectable amounts of milt in males suggests that only half of the 4-year-old Kanagawa strain adults from the INTECH have any possibility of reproduction during the current reproductive season. The other half are either senile or may have completely retarded reproductive activity for this time of the year. These individuals had a characteristic overlapping of lateral scales over the pelvic region, completely covering the white band normally seen in this area (Fig. 7). This senile condition is not normal in captive-reared fish of this age and was more apparent in the females than in males. Interestingly, there appeared to be fewer senile fish in tank KB than in tank

KA, even though both had fish from the same strain and age. The mean condition factor K [100*BW(g)/TL^3(cm)] was 0.65-0.71 for males and 0.67-0.74 for females The 1.5-year-old fish from the Kanagawa strain and the 4-year-old fish from the Junin strain at the INTECH were in better condition as judged from their external appearance, but still looked slightly emaciated (mean condition factor K of 0.70-0.78 for males and 0.78-0.83 for females). Cannulation of representative individuals from the 1.5-year-old Kanagawa strain broodstock in the EHC showed that they were in very good condition and most females possessed vitellogenic and even near mature, pre-ovulation oocytes. These observations suggested that most of the females and males from this broodstock were ready to spawn in the coming weeks. In fact, these observations on the reproductive status of broodstock in the INTECH and EHC were confirmed by the survey of spawning activity, as reported in 1.3.

Fig.7 Appearance of pelvic region of senile fish

Fig.8 Pejerrey with exophtalmia

Fig.9 Pejerrey with superficial hemorrhage

Fig.10 Pejerrey eggs attached to aeration pipes

The reasons for the differences in reproductive condition between the broodstock in the two sites and for the senile condition of the older fish in the INTECH remain unknown, but it might be related to slight differences in rearing conditions at the INTECH and EHC. As regards water quality, there is a strong possibility that well water used at the INTECH is supersaturated with gases, as evidenced by the presence of various individuals with exophtalmia (Fig 8) and superficial

hemorrhage (Fig. 9). Although the fish in the four broodstock tanks did not show such obvious symptoms, it was noted that many of them had what appeared to be an inflammation associated with hemorrhage in the alimentary tract, as shown by the liberation of purulent and bloody fluid from the anus upon application of gentle pressure to the pelvic areas. At this conjecture, it cannot be concluded that such abnormalities are related to gas supersaturation in the rearing water and to the low reproductive activity of the fish, but it is possible that the poor water quality is causing a physiological imbalance and associated low feed intake, and indirectly causing the arrest of reproductive activity. Another possibility is an inadequate feeding regime such as, for instance, administration of nutritionally poor, old, deteriorated, or inappropriately sized diets (too small or too big for the size of the fish), or even frequent changes in feed types. For example, fish at the INTECH received alternatively a newly developed, sinking formula-feed for pejerrey and a floating feed for bullfrog prior to and during the current reproductive season, whereas fish at the EHC consistently received a formula feed for trout. Our own observations in Japan indicate that pejerrey requires an extremely long time to adapt to a change in feed type (e.g. floating vs sinking) and that poor nutritional status prior to the commencement of the spawning season can delay or suppress altogether reproductive activity. It could be also that low reproductive activity at the INTECH is simply a retardation brought about by the lower water temperature (16°C) at this site compared to the EHC (18°C), although it seems improbable that such a minor difference would cause the almost complete arrest of reproductive activity observed at the INTECH. Finally, fish at the INTECH were reared at a salinity of 15 ppt whereas those at the EHC were reared at salinities of 8 ppt (tank A1) and 12 ppt (tank B2). This also does not seem to be a factor since there are reports of sexual maturation and even spawning at salinities up to 20-25 ppt, but it could be worth evaluating further if there is any effect of salinity on reproductive activity in captivity.

1.3- Survey of spawning activity by the broodstock

Purpose: To corroborate information on the degree of gonadal development and the possibility of spawning obtained in 1.2 with the actual observation of spawning activity.

Materials and Methods: Spawning activity was surveyed by daily collection, counting and estimation of fertilization rates of eggs from all broodstock tanks in the INTECH and EHC. Data is available for the period from August 13th to September 9th for the INTECH and from August 13th to September 1st for the EHC (both institutions have continued to send information after the return of this expert to Japan). Information on the 4-year-old Junin strain broodstock from the EHC is based on visual inspection of the tank, as collection and counting was not possible.

Results and Conclusions: Only two spawnings were observed in the four tanks with broodstock at the INTECH, one in tank K1 on August 17th and one in tank KB on September 8th.

Judging from the low number of eggs spawned on each occasion (500 and 7500 eggs from tanks K1 and KB, respectively), both were from a single female in each tank. Fertilization rates in these two egg masses were 28 and 48%, respectively. At the EHC, 4-year-old Junin fish kept in a concrete pond started spawning in the beginning of August, according to accounts from employees. Spawning by the 1.5-year-old Kanagawa strain broodstock at this site began on August 14-15th for group A1, August 17-18th for group B2, and August 30-31st for group A3, and thereafter was observed almost daily (Fig. 10; Table 2).

Table 2. Egg production at the EHC in 2005 (as of September 1st, 2005).

Group	A3			B2			A1		
Date	Number of eggs	Fertilization rate (%)	Eggs per female	Number of eggs	Fertilization rate (%)	Eggs per female	Number of eggs	Fertilization rate (%)	Eggs per female
8/13	NA	NA	NA	NA	NA	NA	-	-	-
8/14	NA	NA	NA	NA	NA	NA	-	-	-
8/15	30000	35	150	NA	NA	NA	-	-	-
8/16	7000	60	35	NA	NA	NA	-	-	-
8/17	6000	NA	30	NA	NA	NA	-	-	-
8/18	6000	60	30	100000	55	133	-	-	-
8/19	NA	NA	NA	NA	NA	NA	-	-	-
8/20	NA	NA	NA	NA	NA	NA	-	-	-
8/21	NA	NA	NA	6400	75	9	-	-	-
8/22	5000	75	25	2200	70	3	-	-	-
8/23	19400	83	97	4000	66	5	-	-	-
8/24	NA	NA	NA	11000	60	15	-	-	-
8/25	17600	75	88	NA	NA	NA	-	-	-
8/26	16600	67	83	NA	NA	NA	-	-	-
8/27	56600	88	283	NA	NA	NA	-	-	-
8/28	NA	NA	NA	NA	NA	NA	NA	NA	NA
8/29	55000	66	275	NA	NA	NA	NA	NA	NA
8/30	76000	80	380	NA	NA	NA	NA	NA	NA
8/31	51000	70	255	NA	NA	NA	22000	NA	110
9/1	63000	80	315	NA	NA	NA	NA	NA	NA
	Total	**Mean**	-	**Total**	**Mean**	-	**Total**	**Mean**	-
	409200	69.9	-	123600	65.2	-	22000	NA	-

Notes: a) Tanks A1 and A3 were stocked with fish from B2 at 8/12 and 8/27, respectively.
b) Tank B2 has been with fish for at least 6 months.
c) NA: not available.

These three tanks usually yielded from thousands to tens of thousand eggs per night starting a few days after stocking with fish. Interestingly, the patterns of total egg production and the number of eggs per female per night in tank A1 suggest the presence of a transient peak of spawning activity a few days after transfer (stocking) to a new tank. A similar phenomenon was observed in tank B2, where 100,000 eggs (estimated to be from 15-20 females) were laid on the night of August 17th, or 2 days after the harvesting and check of the animals in this tank for observation of their reproductive condition. In group A1, spawnings became more consistent toward the end of August. This probably indicates the response to environmental cues by a growing number of females and their entrance in the so-called "reproductive loop" of spring (Strüssmann, 1989). Thus, assuming an output of 5,000-10,000 eggs per spawning per female of the size of those in group A1, it is estimated that about 5-6% of the females were spawning daily in this tank by the end of August. This can be considered as a very good result inasmuch as no manipulation by photoperiod or temperature has been performed in this group. This good egg production, coupled with average fertilization rates of about 65-70% have enabled the obtention thus far of more than 360,000 fertilized eggs at the EHC over a 2-week period.

Overall, these results confirm the inferences on condition and reproductive status of animals made from the visual inspection and cannulation of the females described in 1.2. As previously discussed, the marked difference in reproductive output between the broodstock at the two institutions probably reflects differences in rearing conditions between the two institutions, and this needs to be evaluated further. The apparently increased spawning activity observed in each group a few days after transfer stocking in a new tank or after harvesting could be just a coincidence, but it could be also that stimulation of the fish by netting during the harvesting process is somehow inducing final maturation and spawning. This possibility is worth exploring further for application in artificial or programmed spawning of pejerrey. On the other hand, it is likely that the selection of fish from a common stock at the beginning of their reproductive activity in the EHC led to the greater spawning activity in tank A1. This, in addition to the induced manipulation of broodstock fish by light and temperature treatment are the two most practical ways to obtain synchronization of spawning and improvement of the efficiency of seed production. Nevertheless, the former strategy does not solve the problem of having to rear an excess of broodstock to be able to obtain sufficient number of spawners for seed production.

1.4- Induction of synchronized spawning activity

Purpose: This experiment was originally devised to evaluate the possibility of induction of synchronized spawning by the manipulation of light conditions in the

spawning tanks of the INTECH. However, as reported above, the conditions of the broodstock were not suitable for induction of spawning as virtually all females in the four tanks were emaciated and had markedly retarded gonadal development. Thus, the objective of this experiment became to test the possibility of promoting gonadal recrudescence as opposed to inducing spawning as originally intended.

Materials and Methods: Two experiments were performed at the INTECH. In the first experiment, 4-year-old broodstock from the Junin strain (tank Junin, Table 1) were shifted to a long photoperiod while the adjacent tank (K1), with the 1.5-year-old fish from the Kanagawa strain, remained on a natural photoperiod as the control. Even though the two groups were of different age and strain, both had approximately the same body size (Table 1) and condition, as inferred from the visual inspection of the fish. For photoperiod manipulation, two 100 W fluorescent light bulbs were suspended over the Junin tank and controlled with a timer to provide illumination from 5:00 AM to 9:00 PM (16 hours of light; Fig. 11). Natural light in the control group was from around 8:00 AM to 7:00 PM (11 hours of light per day) at the start of the experiment on August 18th. Evaluation of the effectiveness of the treatment in this case is being performed by the daily observation of the presence of spawning activity.

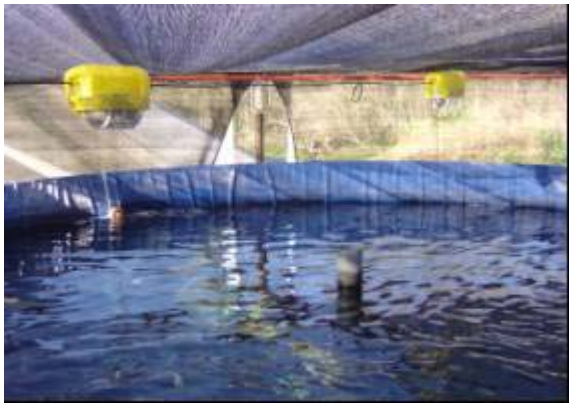

Fig.11 Illumination of Junin tank at INTECH

Fig.12 Selection of fish for experiment indoors

Fig.13 Indoor tanks for photoperiod manipulation

Fig.14 Indoor tanks for photoperiod manipulation

In the second experiment, fish from KA, KB, and Junin tanks were subjected to photoperiod manipulation using eight 20 ton indoor concrete tanks. In total, four tanks were stocked with about 10 females and 10 males from Kanagawa strain each and four with the same number of individuals from the Junin strain. Selection and stocking of the fish was performed on July 30th and the average size of the fish used in this experiment is that indicated in Table 1 (Fig. 12). The four groups in each strain series were subjected from August 13th to either a long photoperiod (18 h light and 6 h darkness, 2 groups) or two short photoperiods as controls (12 h light and 12 h darkness, 6 h light and 18 h darkness). All tanks were covered with thick black plastic to isolate them from external sources of light and a 100 W incandescent bulb and a timer were used to provide the desired photoperiod (Figs. 13 and 14). Evaluation of the effectiveness in this experiment is being made by periodical cannulation of the females and stripping of males for semen collection as in (1). The first observation was performed on August 20th.

Results and Conclusions: As of September 9th, except for the small spawning of about 500 eggs in tank K1 reported in (3), there has been no spawning activity in any of the groups from the first or second experiments. Moreover, cannulation of the females in the second experiment did not reveal any substantial progress in gonadal condition as compared to the beginning of the experiment. Previous laboratory experiments by Strüssmann (1989) indicated that the joint manipulation of the photoperiod and thermal conditions is an efficient method to induce off-season gonadal recrudescence. In that pioneer experiment, fish with regressing or completely inactive gonads at the middle of summer became reproductively active and began spawning within a short period of 2-3 weeks after transfer to a favorable light and temperature regime. The results of this basic research were later tested and confirmed in a fish farm (Yasuda Fish Farm), where transfer of fish from rearing conditions similar to those in the current experiment (e.g., a natural light regime at the end of winter and a nearly constant water temperature around 17°C) to an indoor tank with a long photoperiod (16 h of light) and a temperature around 20-22°C led to a massive, synchronized spawning (Ishida and Yasuda, personal communication). In the current experiment, however, it was not possible to regulate the water temperature in the spawning tanks at the INTECH and it is not known if photoperiod manipulation alone is effective to promote reproductive activity. The lack of a response by the fish so far (3-4 weeks) is not completely unexpected, however, considering the relatively emaciated condition of the animals, the lack of any reproductive activity in the end of winter as indicated in 1.2, and the possible problem with gas supersaturation. Nevertheless, it is still possible that the treatments might induction gonadal recrudescence in the coming weeks or months. Thus, researchers from the INTECH will continue to monitor these fish in the

following months and report their findings for subsequent analysis by this expert. If possible, an attempt should be made to manipulate also the rearing temperature in addition to photoperiod manipulation.

1.5- Evaluation of the effect of salinity on fertilization

Purpose: The fertilization rates of eggs obtained by natural spawning at the INTECH and EHC in the previous years have been low, usually less than 50%. This means that more eggs must be produced to meet yearly seed production quotas than if the fertilization rates were high, and this problem compounds the problem of the lack of synchronization of spawning activity between females. This trend has been confirmed this year at the INTECH, although at the EHC there has been an apparent increase in the mean fertilization rates as compared to last year (Table 2). There are also reasons for concern as the main salt in the rearing water in both institutions is NaCl and high concentrations of this salt inhibit motility of pejerrey spermatozoa (Strüssmann et al., 1994). This has led researchers in both institutions to question whether the range of salinity currently employed in the spawning tanks (e.g. 5-15 ppt) does not impair fertilization rate. To clarify this question, an experiment was performed to evaluate the effects of salinity on the appearance of the eggs, the motility of spermatozoa, and the fertilization rate.

Materials and Methods: This experiment was performed at the EHC with fish from this institution. Eggs from one 1.5-year-old female from the Kanagawa strain were collected by manual stripping after anesthesia. Sperm from one male of the same strain was stripped by abdominal massage and withdrawn into a syringe. Masses of approximately 100-200 eggs were transferred to two series of small plastic jars containing 100 ml of water with salinities of 0, 5, 10, and 15 ppt (Fig. 15). Saline media were prepared with commercially available distilled water and well water from the INTECH (15 ppt). The characteristics of the eggs such as the transparency of the chorion, the occurrence of hydration, and the formation of the blastodisc were compared in one series of jars after 3 hours at room temperature (18°C; Fig. 16). In the other series, 5 µl of sperm was added immediately after introduction of the eggs for evaluation of the fertilization rate. For testing the effects of salinity on spermatozoan motility, sperm was examined under microscope after dilution with the same solutions. The sperm:diluent ratio was approximately 1:5. The intensity of motility was assessed at time 0, 2, and 4 minutes after dilution using an arbitrary scale of motility adapted for pejerrey sperm by Strüssmann et al. (1994).

Results and Conclusions: Eggs immersed in all media had similar rates of formation of the blastodisc (about 50%, 1st series) and fertilization (about 50%, 2nd series) regardless

of the salinity level. However, eggs at the salinity of 0 ppt appeared to be more hydrated, had clearly larger perivitelline spaces, and blastodiscs with irregular shape compared to those at the other salinities (Figs. 17 and 18). It is interesting to note that even eggs without the formation of the blastodisc had a transparent chorion at the salinity of 0 ppt (Fig. 19) whereas those at 5-15 ppt became opaque (Fig. 20). This means that at a salinity of 0 ppt, even eggs that were not completely ripe at the time they were collected, as judged by the absence of a clear blastodisc, underwent hydration and cortical reaction. The similar fertilization rates among the different groups, however, indicate that this feature does not confer any advantage in terms of fertilization. Results of the measurement of spermatozoan motility at different salinities are shown in Table 3.

Fig.15 Salinity series for fertilization experiment

Fig.16 Observation of fertilization rates

Table 3. Effect of salinity on the intensity and duration of motility of pejerrey spermatozoa.

Salinity (ppt)	Motility index*		
	Observation time (minutes)		
	0	2	4
0	5	0	0
5	4.5	1.5	1.5
10	4	2.5	1.5
15	4.5	4	2

* Motility index of Strüssmann et al. (1994): 0 = All spermatozoa are immotile; 1 = Most spermatozoa are immotile and some present lateral vibration; 2 = Most spermatozoa are vibrating or immotile while some present forward movement; 3 = Three classes of spermatozoa can be found in equivalent numbers: spermatozoa moving rapidly, spermatozoa moving slowly or vibrating, and those immotile; 4 = Most spermatozoa move rapidly while some move slowly; 5 = Most spermatozoa display rapid movement; impossible to track the course of any spermatozoa.

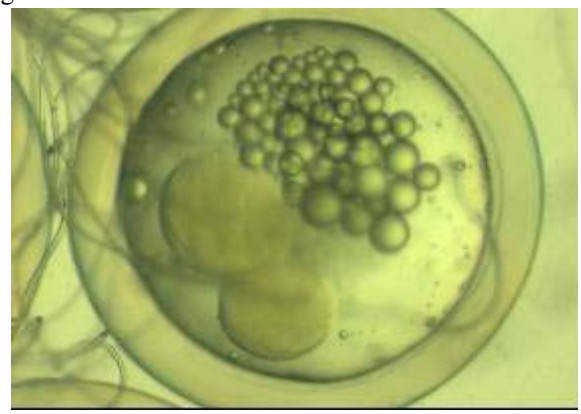

Fig.17 Fertilized eggs at 0 ppt salinity (3 h

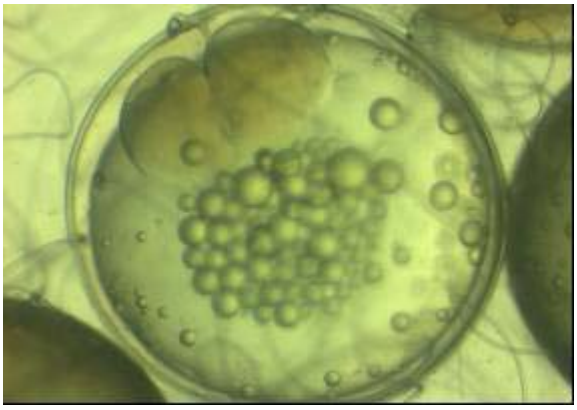

Fig.18 Fertilized eggs at 10 ppt salinity (3 h PSF)

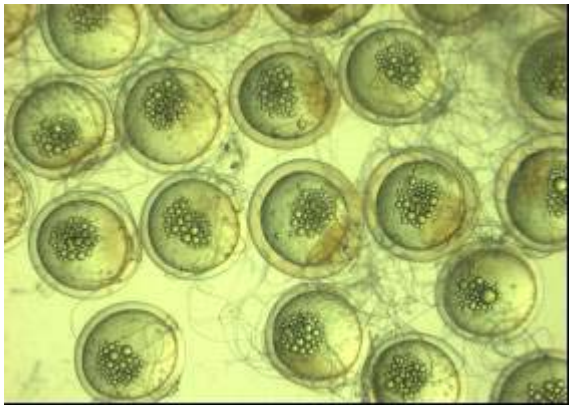

Fig.19 Fertilized eggs at 0 ppt salinity (3 h PSF)

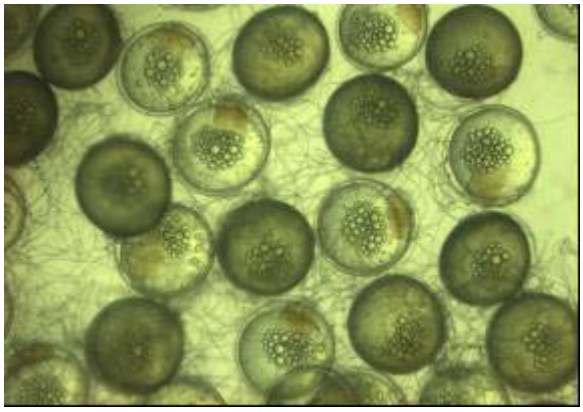

Fig.20 Fertilized eggs at 10 ppt salinity (3 h PSF)

It can be seen that high motility indices were maintained for a longer time with increasing salinity. This result is in agreement with the observation by Strüssmann et al. (1994) that moderate salinities, even with NaCl, promote the motility of pejerrey spermatozoa. Thus, it can be concluded from these experiments that fertilization rates are not impaired by elevated salinity up to 15 ppt. On the contrary, the longer duration of spermatozoan motility with higher salinity, which is probably brought about by the osmotic prevention of the hydration and subsequent lysis of the cells that would otherwise occur at lower salinities, likely means that the spermatozoa had improved chances of reaching the micropyle and fertilizing the eggs. This conclusion is also borne out by the results at the EHC this year, in which mean fertilization rates up to 70% have been obtained at salinities between 8 and 12 ppt. In this context, it remains to be seen why fertilization rates are lower at the INTECH. Two possibilities are the differences in rearing water quality (gas supersaturation) and fish condition, as noted previously. Another difference between the rearing conditions in the two sites is the markedly reduced water flow (exchange) rate in the tanks at the EHC in comparison to the INTECH (this has been done purposely to reduce the amount of salt in the supply water as it increases with increased water uptake in the former institution). Thus, further experiments should compare the effect of water exchange rates on the fertilization rates in spawning tanks.

1.6- Evaluation of methods for marking of seeds for release

Purpose: Seeds for release must be marked to distinguish them from wild ones. This is necessary for future recapture of these individuals and the assessment of the effectiveness of the (re)stocking programs, and to obtain information such as optimum release size, location and time. At present, there is no definite method for the marking of pejerrey juveniles. Earlier studies by Kanagawa Prefecture Fisheries Experimental Station and by researchers in the INIDEP (Argentina; Brown and Fuentes, personal communication), and more recently by researchers at the INTECH, have focused

on the marking of otoliths with compounds (e.g. Oxytetracycline, Alizarin) that fluoresce under illumination with certain wavelengths. This method, while practical from the perspective of marking, is time consuming during subsequent analysis since it requires the extraction of the otoliths. It also requires expensive and sophisticated equipment (e.g. a fluorescent microscopy). Other studies by the Kanagawa Prefecture Fisheries Experimental Station and this expert have used the subcutaneous implantation of fluorescent silicone elastomeres, which allow the immediate identification of the fish. However, since they are injected in the muscle, there is always the possibility of inadvertent consumption of the silicone elastomere by anglers. Thus, this experiment was conducted to evaluate the suitability of new marking methods under development for other species for application in the marking of pejerrey juveniles. These methods tested in this study are the heat (or cold branding), as is being tested in flounder, and the subcutaneous implantation of fluorescent silicone elastomere in the base of the pectoral fin, as its being done on puffer fish.

Materials and Methods: This experiment was performed at the INTECH with 10-months-old juveniles brought from the EHC. The range of size of fish was 6.8 to 12.9 cm in body length and 3.6 to 27.9 g in body weight. In total, 16 fish were used for heat branding, 14 for cold branding, and 10 for silicone elastomere implantation. Fish were anesthetized in 100 ppm benzocaine. For heat branding, a standard solder (150W) with a tip diameter of about 5 mm was heated to equilibration and applied for 5 seconds to one or two locations on the dorsolateral wall of the fish near the dorsal fin (Fig. 21). For cold branding, the tip of the same solder was dipped in liquid nitrogen until thermal equilibration and marks were applied for 5, 10, or 20 s in one or two locations as for heat branding (Fig. 22). Fish were marked on different locations (right x left, anterior x posterior) to identify different treatments. The wound caused by the heat and cold branding was disinfected with Isodine solution (Fig. 23). Two colors of silicone elastomere were used for implantation in the transparent base of the pectoral fins on both sides of the fish. Half of the fish was implanted with blue elastomere and the other half with pink. In both cases, fish were implanted also in one side of the dorsolateral wall above the pectoral fin as a control in case of the loss of the elastomere implanted in the base of the pectoral fin (Fig. 24). In all types of marking, fish were allowed to recover from the procedure in a 200 L tank with flowing water at 17°C and a salinity of 15 ppt. The tanks continue to be monitored for mortality and the visibility of the marks as of today.

Results and Conclusions: All marks were clearly visible on the next day (Figs. 25, 26, 27, and 28). The wound caused by the heat and cold branding healed completely within two weeks of the treatment. Mortality was limited to 2 fish out of 16 in the heat branding group and 1 out of 14 in the cold branding group as of September 9th. No mortality has been observed in the silicone elastomere-implanted fish.

Fig.21 Heat branding of pejerrey juveniles

Fig.22 Cold branding of pejerrey juveniles

Fig.23 Disinfection with Isodine after branding

Fig.24 Implantation of silicone elastomere

Fig.25 Heat-branded pejerrey juveniles

Fig.26 Cold-branded pejerrey juveniles

Fig.27 Silicone elastomere-implanted juvenile

Fig.28 Silicone elastomere-implanted juvenile

The implantation of silicone elastomere in the base of the pectoral fin is advantageous over that in the dorsal muscle in which it is not an edible part. Thus, the risk of inadvertent consumption by anglers can be minimized by this method. The three methods therefore appear promising and the final choice should be based on the consideration of the time and skills involved in marking. As a reference, it was estimated that it took about 25-30 seconds per fish for the marking with cold branding and elastomere implantation and 15-20 with the heat branding. Observations should be continued in the following months to evaluate further the long-term visibility of the marks. However, there are currently over 30,000 seeds produced last year that still await marking and release. Any of the methods could be applied for these seeds but, for this purpose, it is recommended that, whatever the choice of marking method, it be applied in conjunction with another traditional method (e.g. otolith marking, coded-wire tags, etc) pending the results of the long-term evaluation to ascertain the usefulness of each of the proposed methods.

2- Recommendations

2.1- Broodstock Management

2.1.1-The first production cycle should start as early as possible in the end of winter/beginning of spring (even if this means having to rear larvae on *Artemia* nauplii for the first weeks because of the inability to raise plankton).

Advantages: This will allow seeds obtained in the first cycle to be released at a time when plankton is naturally blooming in the lagoons (instead of at the end of summer, when plankton is declining, as is currently done) and therefore lead to higher survival and growth rates (which will probably compensate the higher cost incurred in their production due to the use of *Artemia*). An additional advantage is the attainment of yearly production quotas earlier in the season. This should alleviate the pressure towards the last months of summer, which is the period of summer vacations and shortage of personnel.

Requirements: (a) Off-season spawning, which can be achieved by selection of broodstock and manipulation of the light and temperature conditions for broodstock rearing, as indicated in 2.1.2 and 2.1.3. (b) Ability to produce plankton at this time of the year, or to purchase cysts of *Artemia* and/or high quality artificial feeds for use when natural plankton is not available.

2.1.2-Broodstock should be selected at the start of the spawning season (beginning to middle of August) and separated into smaller groups according to the probability of spawning in the following weeks or months. This is most necessary for the youngest and oldest animals, where the highest variability in reproductive conditions occurs. Criteria for

"immediacy" of spawning and/or culling will be the health (good nutritional conditions, absence of disease or deformities) and reproductive (gonadal development, as judged from the bulging of the belly and presence of milt in females and males, respectively) status of the animals.

Advantages: (a) This will allow the prediction of the time of spawning and egg production more accurately. (b) Possibility of induction and synchronization of spawning among fish if necessary (because it is easier to manipulate the light, temperature and salinity conditions in smaller tanks). (c) Likely obtention of higher fertilization rates due to the control of salinity in smaller tanks and removal of the "disturbance effect" of non-reproducing animals. (d) Precise monitoring of the reproductive status of fish, leading to a better understanding of processes such as reproductive aging (senility) and response to environmental cues, which in turn might allow the development of early, mean, and late spawning broodstock groups.

Requirements: Clear criteria to be used during screening/culling of animals for their health and reproductive status.

2.1.3-Spawning should be induced by the manipulation of light and/or temperature conditions in the broodstock rearing tanks. Animals for this purpose should be selected from among the larger group of broodstock, as indicated in (2.1.2).

Advantages: (a) Same advantages listed in (2.1.2) as well as the attainment of production quotas using fewer broodstock fish, which means reduced maintenance cost and consequently of seed price. (b) In addition, since more fish will respond to the sudden change in environmental conditions than under natural changing conditions, it is possible to obtain seeds from and between fish that normally will not overlap in their reproductive activity. This will ensure that the seeds produced will have maximum genetic variability while at the same time making possible to rear (and manipulate) the minimum number of broodstock possible.

Requirements: (a) Rearing facilities with controlled light conditions (minimum) and, if possible, also an indoor (glass house) 5-10 ton, recirculated water tank to be able to rear the broodstock at warmer temperatures when necessary (but without the need for artificial heating) and at controlled salinity (because moderate salinity prolongs sperm motility and hence promotes fertilization rates). (b) Knowledge on the effects of environmental factors (light, temperature, food amount and quality, and possibly also of salinity, water flow, atmospheric pressure, lunar cycles) on reproductive status. (c) Testing of the efficiency of joint environmental and endocrine (hormonal) stimulation of reproductive activity.

2.1.4-Broodstock fish should be fed high quality artificial feeds for at least 2-3 months before the start of the spawning season. Given the lack of a standard diet optimized for pejerrey at this moment, it is suggested that pejerrey should be fed with trout diets, which are more

standard in composition than the other alternatives available in the domestic market or under development. Trout diets provide adequate levels of protein and lipids to sustain the heavy nutritional demands of broodstock fish prior to and during spawning. Attention should be also placed on choosing the right size of feeds in relation to the size of the fish.

2.1.5-Broodstock for seed production should be renovated every two years with fertilized eggs collected from natural populations that are within the geographic area where the produced seeds will be released. The reasons are as follows. (a) Fertility (reproductive ability) peaks at 2-4 years in pejerrey, particularly under the project site conditions. (b) The genetic variation in Kanagawa stock (or any other stock, for that matter) will probably deviate and end up being not representative of the genetic makeup of natural populations, under continuous, multi-generation rearing. (c) Captive-reared broodstock also serves as a buffer against the temporal or spatial inability to locate wild sources of seeds (Note: "lessons from the past": while it could be argued that, if constant renovation is necessary, than it would be easier to take directly from wild populations every year, it must be understood that a) reproduction activity in wild stocks is unpredictable, therefore making them unreliable sources of seeds, b) the seasonality of reproduction in wild stocks probably does not support an extended seed production scheme with 2- or 3-production cycles per year.)

2.2- Miscellaneous recommendations

2.2.1- The current facilities could be used to have up to three production cycles per year (season) (assuming a cycle of about 2-3 months with an overlap of a few weeks between cycles, which can be done in different tanks)**.**

Advantages: This will allow maximum return of investment in facilities and personnel and the advantages listed in (2.1.1).

Requirements: (a) Selection of broodstock and induction of off-season and synchronized spawning as discussed in 2.1.2 and 2.1.3. (b) Cysts of *Artemia* and/or high quality artificial feeds for use when natural plankton is not available.

2.2.2- The following improvements are suggested for the rearing facilities of the INTECH and EHC. Improvements that apply to both the INTECH and EHC are as follows. (a) Rearing areas should be equipped with paved corridors adjacent to the tanks that allow the passage of a heavy-load caster (to carry a tank of up to 300 L to be used during transport of fish between tanks and when loading them onto a truck. (b) One edge of the corridor should border an access area where a transport truck can park to load/unload fish. (c) Aeration outlets (with valve) should be installed in strategic areas near the tanks for use during sampling, transport, etc. (d) Indoor tanks for use during photoperiod and temperature

manipulation of broodstock for spawning as indicated in 2.1.3. Improvements that apply to the INTECH are as follows. (e) A system to reutilize the rearing water from the 6 outdoor tanks for the earthen ponds available at this institution (see 2.2.4). (f) Paddlewheels or any other equipment to vigorously aerate the primary water reservoir, to eliminate excess gas (see 2.2.5)

2.2.3- Experiment with different methods for collection of eggs from the tanks (such as the use of spawning mats in the bottom, etc.). This is necessary to improve the efficiency of the egg collection method, which is very inefficient at present.

2.2.4- Experimentally raise pejerrey in the earthen ponds in the INTECH. This appears necessary to a) obtain preliminary information on the feasibility of semi-intensive cultivation, and b) give personnel much needed hands-on experience on rearing fish to marketable size (future extension workers in aquaculture). This can be achieved at a relatively low cost by using existing earthen ponds in the INTECH and by reusing rearing water from the existing broodstock tanks.

2.2.5- Measure dissolved gases (especially nitrogen) and other substances (pollutants, heavy metals, etc) in the rearing water. (In the INTECH, there are indications of a water quality problem, most probably gas supersaturation). This is an urgent task!

2.2.6- Obtain an agreement/pledge from persons, companies, or organizations receiving seeds through the project. It is desirable that they a) recognize the number, size, date of delivery etc of seeds, b) agree not to resell or give away fish without consent from the provider of seeds, c) agree to allow the conduction of periodic surveys for monitoring of the growth and survival of the stocked seeds, d) agree to disclose any information on the site (water conditions etc), harvesting and commercialization of the produce which might be of relevance for the evaluation of the effectiveness of stocking and of aquaculture in general, and e) release the provider of seeds of any responsibility such as in the case of losses, liability claims, environmental degradation etc incurred as a result of the stocking with seeds.

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Strüssmann, C.A., Renard, P., Ling, H., and Takashima, F. 1994. Motility of pejerrey *Odontesthes bonariensis* spermatozoa. *Fisheries Science* 60(1):9-13.

別添資料３：遺伝形質解析分野英文レポート

Outline of activities and recommendations from evaluation mission on the JICA Project of Pejerrey Propagation and Aquaculture in Argentina (August 11th-22nd, 2005)

Dr. Takashi Sakamoto
Associate Professor
Tokyo University of Marine Science and Technology

Field of expertise:

Population genetic analysis

Field of activities:

Genetic evaluation of broodstock and natural populations

Institutions visited:

Instituto Tecnológico de Chascomús (INTECH)

Estación Hidrobiológica de Chascomús (EHC)

Summary of activities and recommendations:

The main activity conducted during my visit to Argentina was the genetic evaluation of broodstock maintained at the INTECH and the EHC and of surrounding natural populations, particularly of Lake Chascomús, together with researchers from the INTECH. The goal was the obtain basic information on the degree of genetic variation of the various populations and estimate the genetic differences between them to serve as a reference for decisions involving the release of artificially produced seeds from any particular broodstock in natural water bodies. This analysis was conducted using a technique called microsatellite marker analysis.

In general, microsatellite markers are very sensitive and informative genetic markers that can detect very small genetic differences among populations. Because of this sensitivity, it is desirable to collect genetic data using as many markers as possible (at least three), to prevent a disproportionate effect on the conclusion from any single positive or negative result. However, in this study, we could only collect data sets for two markers because of difficulties in the use of a new electrophoresis equipment purchased for this project. Moreover, only the first set, using the microsatellite marker Obo01TUF, contains information for all five populations under study (Chascomús, Kanagawa, Junin, Salada Grande and Chasicó). The second data set, obtained with two microsatellite markers (Obo01TUF and Obo02TUF), has

genetic information for only two of the populations (Chascomús and Kanagawa). This limited amount of genetic information is not enough for a conclusive population analysis. The genetic differences among these populations were analyzed anyway, taking in consideration the urgent need for information on the genetic status of seeds from the Kanagawa strain produced at the EHC. Genetic analysis of the first data set using the Genepop software revealed significant genetic differences among all pairs of populations. We were also able to detect a significant genetic difference between the Chascomús and Kanagawa populations using the second data set and the Arlequin genetic analysis software. The Genepop software was used also to calculate pairwise F_{ST} values for estimation of the genetic divergence between populations (Table 1). The F_{ST} value between Chascomús and Kanagawa was 0.0637 whereas that between Chascomús and Chasicó was 0.0479. The smallest F_{ST} value encountered was 0.0236 (between Chasicó and Junin). In our discussions (Dr. C.A. Strüssmann, Dr. G.M. Somoza, Lic. G. Berasain and Dr. T. Sakamoto), we have concluded that the low F_{ST} values between populations such as Chasicó and Junin, or between Chascomús and Chasicó, are probably related to previous programs of pejerrey stocking. For instance, it is common knowledge that pejerrey stocking programs have been carried out in Argentina since the beginning of the 20th century, and that these have used alternatively various lagoons as source of seeds for introduction. Unfortunately, there has never been any genetic study of wild populations in Argentina, so pejerrey stocking programs have been conducted without consideration for the source and destination of seeds. This means that it is now impossible to estimate the original genetic population structure of pejerrey prior to stocking programs from sampling of wild specimens.

Nevertheless, it is important to recognize that there is still a fairly large degree of genetic divergence between the various groups, which suggests the existence of subpopulations with limited gene flow between themselves. In this regard, the Kanagawa strain was derived from the pejerrey population of Lake Chascomús, which has no known history of introduction of seeds from other populations prior to the sending of fertilized eggs to Japan in 1966, and has been isolated ever since. This strain was never allowed to interbred with other pejerrey populations available in Japan and has never been subjected to any kind of artificial modification of its genetic makeup. Thus, it is very likely that the Kanagawa strain represents the genetic constitution of the pejerrey population of Lake Chascomús in 1966, and, in this regard, may prove invaluable to restore the original pejerrey strain to this lake. More importantly, however, the results of this analysis show that the Kanagawa strain is no more different from the other strains available for this study, and which have been used in previous stocking programs for pejerrey, than these strains compared to each other. For example, pejerrey stocking programs in Lake Chascomús and many other water bodies throughout

Argentina since the demise of the Chascomús population have been carried out using pejerrey seeds from Junin and Salada Grande, among other sources (Lic. Berasain, personal communication), which have the highest level of genetic divergence among the populations studies (F_{ST} value of 0.2658; Table 1). In this context, it is our (Dr. C.A. Strüssmann and Dr. T. Sakamoto) conclusion that there are no fundamental differences in carrying out restocking programs with the Kanagawa strain as far as genetic divergence between the populations is concerned. However, to avoid the gradual loss of genetic diversity often encountered in multigenerational rearing of broodstock as well as to conserve as much as possible the genetic variability of wild populations, it is recommended that future stocking programs try as much as possible to renew and diversify the broodstock every few years using as a source wild natural populations with a genetic constitution as close as possible to those in the area where restocking is to be carried out. As a basis for these activities, it is of foremost importance to continue the study of the genetic diversity of Argentine pejerrey populations.

Table 1. Population pairwise F_{ST} estimates among six populations of wild (w), F_1 (f) and hatchery (h) pejerrey using microsatellite marker (Obo01TUF)

	Chascomús (w)	Kanagawa	Junin	Salada Grande
Kanagawa (h)	0.0637	-	-	-
Junin (f)	0.1062	0.2521	-	-
Salada Grande (f)	0.0822	0.0842	0.2658	-
Chasicó (f)	0.0479	0.1825	0.0236	0.1779