サバ科雑種宿主によるクロマグロ精子の生産

博士学位論文

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2020 年度

(2020 年 9 月)

東京海洋大学大学院

海洋科学技術研究科

応用生命科学専攻

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緒言

我が国はクロマグロ（*Thunnus orientaris* および *Thunnus thynnus*）の世界最大の消費国であり、2017 年における我が国のクロマグロ供給量は総計 4.88 万トンと世界全体の生産量の 6 割以上を占めている（水産庁, 2019）。本マグロとも呼ばれる本種は、その肉質の良さからマグロ属の中でも最も高値で取引されており、世界中で日本への輸出に向けたクロマグロの漁獲や養殖が盛んにおこなわれている。また、最近では健康食ブームや寿司人気の高まりによって、欧米やアジア諸国でのマグロの消費が拡大しており、今後もこの需要拡大は続いていくと予測されている。

このような需要増加に伴い、クロマグロ資源の減少が大きな問題となっている。現在、クロマグロは太平洋クロマグロ、大西洋クロマグロともに国際自然保護連合（International Union for Conservation of Nature and Natural Resources: IUCN）によって絶滅危惧種に指定されている（IUCN, 2019）。特に、太平洋クロマグロは日本の周辺海域内に産卵場が存在するため（Shimose, 2019）、我が国が率先して本種の資源量増加に向けた策を講じる必要がある。北太平洋マグロ類国際科学委員会（International Scientific Committee for Tuna and Tuna-like Species in the North Pacific Ocean: ISC）が 2018 年に更新した太平洋クロマグロの資源評価によると、最近年（2016 年）の親魚資源量は約 2.1 万トンであると推定され、歴史的最低値を記録した 2010 年の約 1.2 万トンから緩やかな回復傾向にあることが示された（ISC, 2018）。したがって、近年の国際的な取り決めに基づく資源管理の強化によって、太平洋クロマグロの資源は増加傾向にあると考えられるが、親魚資源量の歴史的中間値が約 4.3 万トンであることからも、依然として太平洋クロマグロの資源水準は低位にあるとされている（水産庁, 2019）。

現在、クロマグロ類を含むカツオ・マグロ類は 5 つの地域漁業管理機関（Regional Fisheries Management Organization: RFMO）によって世界中の全ての海域

で資源管理が行われている。我が国は 5 つすべての RFMO に加盟しているが、中でも特に重要なのが日本の排他的経済水域を管理する中部太平洋マグロ類委員会（Western and Central Pacific Fisheries Commission: WCPFC）である。2014 年に開催された WCPFC の年次会合では、太平洋クロマグロの親魚資源量を 2024 年までに歴史的中間値である約 4.3 万トンまで回復させることを当面の目標とし、30 kg 未満の小型魚の漁獲量を 2002 年-2004 年の平均水準から半減させる等の管理措置が採択された（WCPFC, 2014）。我が国ではこの採択を受けて、2015 年の 1 月より 30 kg 未満の小型魚の漁獲上限を 2002 年-2004 年の平均漁獲実績 8,015 トンから半減し、4,007 トンとすることを発表した。このような小型魚の漁獲規制が施行された要因の 1 つとして、0 歳から 3 歳までの未成魚の高い漁獲圧があげられる。ISC によると太平洋クロマグロの全漁獲尾数に対する 3 歳以下の未成魚の割合は 97.9%であると推定され、中でもヨコワと呼ばれる 0 歳魚の割合は 73.2%を占める（ISC, 2018）。これらの未成魚は食用として利用されるほか、養殖原魚としての利用も多い。我が国ではクロマグロ養殖産業の急速な拡大に伴って、曳き縄による養殖用ヨコワの漁獲が増加し、2000 年代以降では 0 歳魚の総漁獲尾数の 1/4 程度が養殖用として利用されている（升間, 2015）。生簀へ活け込まれた全長 20-30cm、体重 100-500 g のヨコワは 2-3 年間（2 年で約 25-30 kg、3 年で約 50-60 kg）養成された後、市場へ出荷される。また、メキシコで漁獲された未成魚（主に 2 歳魚）はほとんどが養殖原魚として利用されている（水産庁, 2019）。

他方、大西洋クロマグロでは、資源量の減少を引き起こした要因の 1 つが産卵回遊にきた成魚を旋網によって漁獲し、養殖原魚として利用する蓄養産業の拡大であると考えられている（Metian et al., 2014; Die, 2016）。1990 年代から、主に地中海諸国、オーストラリア、メキシコにおいて大西洋クロマグロの“畜養（fattening）”が盛んにおこなわれてきた。畜養とは、旋網などで漁獲した 20-60 kg の天然種苗を 6-7 カ月間生簀で飼育し、給餌によって可食部への“脂の乗り”を良くした後に出

荷する養殖様式のことである（Ottolenghi et al., 2004）。この畜養によって日本人が好むトロが多くとれるようになったため、日本が高値で大西洋クロマグロを輸入するようになった。これにより、養殖場の建設が相次ぎ、産卵回遊にきた成魚が過剰漁獲されたことが、大西洋クロマグロ資源量の減少に拍車をかけたといわれている（Metian et al., 2014; Die, 2016; Porch et al., 2019）。現在では、大西洋マグロ類保存国際委員会（ICCAT）による資源管理の強化によって大西洋クロマグロの資源量は回復傾向にあるが（ICCAT, 2017）、我が国でもこの前例にならい、天然種苗の過度な利用を避ける様な対策が取られ始めている。

このような漁業規制が強化されていく中で、天然種苗に依存しない完全養殖技術への期待が高まっている。クロマグロの完全養殖技術は近畿大学によって2002 年に構築され（Sawada et al., 2005; Masuma et al., 2011）、人工種苗を用いたクロマグロ養殖がすでに展開されている（熊井, 2010）。しかし、飼育下で産卵可能なクロマグロ親魚は一般的に 3 歳から 5 歳で初産を迎え、大型個体では全長 3 m、体重 500 kg にも達するため、親魚の養成には大型の海面生簀が必要なうえ、親魚の維持には大量の餌料や多大な労力を要する（Seoka et al., 2007; Masuma et al., 2008）。また、海面生簀で飼育したクロマグロの性成熟の進行や産卵回数、産卵頻度、産卵寄与率、受精卵数および卵質は、年や地域により大きな変動があることが報告されており（升間, 2011）、受精卵の安定供給が課題となっている。

最近では、水産研究・教育機構によってクロマグロの安定採卵技術の開発を目的として、直径 20 m×深さ 6 m、容量 1,880 m^3 の循環ろ過式大型陸上水槽が西海区水産研究所のまぐろ増養殖研究センターへ設置され、2014 年に調光および調温による環境制御によってクロマグロを産卵させることに成功している（樋口ら, 2015; Higuchi et al., 2019）。このような陸上水槽を用いた環境制御による産卵誘発は安定的に採卵できる可能性がある反面、初期設備投資およびランニングコストが莫大である。このように完全養殖技術の確立以来、クロマグロの人工種苗生産技術は

急速に発展しているが、クロマグロという巨大な生物を飼育する以上、上述のように親魚の管理に莫大なコストや労力、スペースが必要となることは避けられない。

そこで、本研究室ではサケ科魚類において開発された代理親魚技法をクロマグロへ応用することを考えた。代理親魚技法とは、ドナーとなる個体の生殖腺から得た生殖幹細胞を、宿主魚の仔魚の腹腔内へ移植し、成熟した宿主魚にドナー由来の配偶子を生産させる技術である（Yoshizaki and Yazawa, 2019）。実際に本技法を用いて、ニジマス *Oncorhynchus mykiss* の生殖幹細胞を、ニジマスの近縁種であるヤマメ *Oncorhynchus masou* の仔魚へ移植することで、成熟したヤマメ代理親にドナー由来のニジマス配偶子を生産させることに成功している（Okutsu et al., 2007）。また、現在では様々な海産魚種へ代理親魚技法が応用され、アジ科（Morita et al., 2012, 2015）、フグ科（Hamasaki et al., 2017, Yoshikawa et al., 2018b）、ニベ科（Yoshikawa et al. 2017, 2018a）でドナー由来の配偶子を生産する宿主魚の作出に成功している。

そこで、この代理親魚技術をクロマグロへ応用できれば、クロマグロの生殖腺から得た生殖幹細胞をサバなどの小型近縁種へ移植することにより、成熟したサバ代理親からクロマグロ種苗を生産することが可能になると期待される。クロマグロの代理親候補であるサバ科マサバ *Scomber japonicus* は、雌雄ともに全長約 30 cm 体重約 300 g、満 1 歳で成熟することが明らかにされている（石橋ら, 2007）。代理親魚技法により、マサバからクロマグロの配偶子を得ることができれば、親魚を陸上の小型水槽で飼育することが可能であるため、海面生簀で行われるクロマグロの親魚養成に必要な広大なスペースや給餌にかかる莫大なコストを大幅に削減することが可能となる。また小型でハンドリングが容易なマサバは、ホルモン投与による産卵誘発技術が確立されているため（Shiraishi et al., 2005）、計画的かつ安定的なクロマグロ受精卵の採卵が可能となるのみならず、陸上水槽での水温や日長の制御とホルモン投与を組み合わせることで、早期採卵等の非産卵期における種苗生産が可能になると期待される。さらに、次世代のクロマグロ養殖産業には有用系統の

作出が必須であるが、クロマグロの成熟には最低でも 3 年を要し、世代時間の長さがクロマグロの家魚化･育種において問題となる。そこで、1 年で成熟するマサバを代理親とすれば、世代時間の短縮によってクロマグロ育種の高速化が可能になる。以上の背景から、当研究室ではクロマグロ代理親魚技法の構築を目指し、すでにマサバを宿主に用いた移植技術を開発している（Yazawa et al., 2010, 2013）。

代理親魚技法において正常に成熟する宿主を用いると、移植細胞由来の配偶子に加えて宿主自身の配偶子が大量に生産されるため、次世代のほとんどが宿主由来となることが問題となる。そのため、ドナー由来のクロマグロ配偶子のみを生産するサバを作出するためには宿主の不妊化が必須となる。しかし、現在までにサバ科魚類において安定的かつ大量に不妊魚を作出可能な技術の開発には至っていない。これまでの先行研究では、受精卵への温度･圧力処理による三倍体化（Okutsu et al., 2007; Yoshizaki et al., 2010; Lee et al., 2013; Yoshikawa et al., 2017; Hamasaki et al., 2017; Seki et al., 2017）や生殖細胞の生存に必須な遺伝子である *dead end* 遺伝子（Weidinger et al., 2003）の機能阻害が不妊化法として利用されてきた（Saito et al., 2008; Yoshizaki et al., 2016; Li et al., 2017; Octavera and Yoshizaki, 2019; Marinović et al., 2019）。しかし、クロマグロの宿主候補となるサバ科魚類は初期減耗が著しく、これら受精卵への処理が必要な不妊化方法は、宿主の生残に多大な影響を与えることから、不妊宿主の大量生産には不向きである。したがって、クロマグロ代理親魚技法を実現するために、宿主魚の生残に影響を与えない方法による不妊宿主の安定生産が必須である。そこで本研究では受精卵への処理が不要な宿主魚の不妊化方法として種間交雑に着目した。

古くから農業や畜産業の分野では雑種強勢効果を期待して多くの雑種が作出されてきた。その中で、メスのウマとオスのロバの雑種であるラバが不妊である例など、両親種の組み合わせによっては雑種が不妊性を示すことは、様々な生物種で知られている（Coyne and Orr, 2004; Maheshwari and Barbash, 2011）。水産分野にお

いても育種技術の一つとして種間交雑は様々な魚種で実施され、現在までに多くの不妊雑種が作出されている（Bartley et al., 2001; Rahman et al., 2013）。特にサケ科では様々な組み合わせの種間交雑が試みられており、例えばカワマス *Salvelinus fontinalis*♀×サクラマス *Oncorhynchus masou*♂雑種やスポーツフィッシングの対象魚として有名なタイガートラウト（ブラウントラウト *Salmo trutta*♀×カワマス♂雑種）も不妊の雑種となる（Arai, 1984; Chevassus, 1979; Gray et al., 1993）。そのほかにも淡水魚の不妊雑種はメダカ科（Hamaguchi and Sakaizumi, 1992）、コイ科（Yamaha et al., 2003）、チョウザメ科（Linhartová et al., 2018）、トゲウオ科（Takahashi et al., 2005）、ナマズ科（Ponjarat et al. 2019）など様々な魚種で知られている。

また、雑種作出の報告は淡水魚に比べ少ないが、海産魚においても不妊雑種となる両親種の組み合わせとして、タイ科のマダイ *Pagrus major*♀×クロダイ *Acanthopagrus schlegelii*♂雑種およびマダイ♀×チダイ *Sparus sarda*♂雑種（Murata et al., 1997）やニベ科のニベ *Nibea mitsukurii*♀×シログチ *Pennahia argentata*♂雑種（Yoshikawa et al., 2018）、ヒラメ科の Yellowtail flounder *Pleuronectes ferrugineus*♀× Winter flounder *Pleuronectes americanus*♂雑種（Park et al., 2003）などが知られている。

多くの場合、雑種では両親種の異なる染色体構成が引き起こす減数分裂の異常によって不妊となることが知られている。雑種生殖細胞の減数分裂時における染色体の挙動を詳しく調べた例として、哺乳類では実験用近交系マウス C57BL/6 *Mus musculus domesticus*♀×ヨーロッパハツカネズミ *Mus spretus* 雑種（Matsuda et al., 1992）や、ジャンガリアンハムスター *Phodopus sungorus*♀×キャンベルハムスター*Phodopus campbelli*♂雑種（Ishishita et al., 2015）、鳥類ではアヒル *Anas platyrhynchos*♀×バリケン *Cairina moschata*♂雑種（Islam et al., 2013）、また魚類においてもニホンメダカ *Oryzias latipes*♂×ジャワメダカ *Oryzias cuvinotus*♀雑種（Hamaguchi and Sakaizumi, 1992）の報告があるが、いずれの雑種においても不妊

の主たる原因は、第一減数分裂中期までに生じる減数分裂の停止としている。また、両親種間の染色体構造の違いによるザイゴテン期やパキテン期での相同染色体の対合異常およびそれに伴う染色体組換えと染色体分離の異常によって減数分裂の停止が引き起こされると報告している。

したがって、3 セットの染色体を保持することに起因する減数分裂異常によって不妊となる三倍体と同様に、不妊雑種においても生殖腺体細胞は正常であることが予想され、代理親魚技法の宿主として利用可能であると考えられる。実際に、Yamaha ら（2003）はキンギョ *Carassius auratus*♀×コイ *Cyprinus capio*♂不妊雑種を宿主として、正常なキンギョ胚から採取した pre-PGC を含む細胞集塊を移植することで、ドナーに由来するキンギョ精子のみを生産させている。また、Wong et al. 2011 ではゼブラフィッシュ *Danio rerio*♀×パールダニオ *Danio albolineatus*♂不妊雑種を宿主として、wTg（*vasa:DsRed2-vasa;bactin:EGFP*）ゼブラフィッシュの卵原細胞移植によって、ドナーに由来する精子のみを生産させている。さらに、最近ではニベ♂×シログチ♀雑種が生殖細胞欠損型の不妊雑種となることが明らかにされ、本雑種を宿主としてドナー由来のニベ配偶子を生産可能であることが示されている（Yoshikawa et al., 2018a）。以上のように種間交雑による不妊化は、魚類での知見も多いうえ、代理親魚としての利用例も報告されている。しかし、不妊となる両親種の組み合わせの明確な規則性は不明なうえ、サバ科魚類において不妊雑種の作出例は皆無である。そこで、本研究ではクロマグロの代理親として利用可能なサバ科の不妊雑種を作出することを目的とした。

第 1 章ではゴマサバ *Scomber australasicus* とマサバに着目し、ゴマサバ×マサバ雑種の生残性と妊性について調べた。さらに、本研究ではクロマグロの有力な宿主候補であると期待されているスマ *Euthynnus affinis* の種間交雑にも着手した。代理親魚技法によって宿主にドナー由来の配偶子を高効率で生産させるためには、ドナー種に合わせて適切な宿主種を選択することが重要である。一般に、宿主種と

ドナー種が遺伝的に近縁であるほど宿主のドナー由来配偶子の生産効率が高い（Bar et al., 2016; Yoshizaki and Yazawa, 2019; Goto and Saito, 2019）。また、産卵適水温等の繁殖に関わる生理学的特性が宿主種とドナー種で類似しているかどうかも、宿主によるドナー由来配偶子の生産効率に関わる重要な要素であると考えられる。スマの属するサバ科スマ属は、クロマグロが属するサバ科マグロ属と遺伝的に最も近縁な関係にある分類群であり（Miya et al., 2013）、インド太平洋の熱帯から亜熱帯に生息するスマは産卵適水温等の生理学的特性がクロマグロと類似している（Yesaki, 1994）。したがって、スマの不妊雑種を作出することができれば、クロマグロ配偶子を高効率で生産する宿主になることが期待される。スマ雑種の作出には近縁種の凍結精子が必要となるが、スマの近縁種であるカツオ・マグロ類は大型の回遊魚であり、精子の採取は船上や漁港といった凍結精子の作製に不向きな野外環境で実施することになる。そこで、まず第 2 章では野外でも簡便にカツオ・マグロ類の凍結精子を作製可能な手法を開発した。そして第 3 章では、スマ *Euthynnus affinis*♀から得た卵に対して、3 属 4 種（カツオ属カツオ *Katsuwonus pelamis*、ソウダガツオ属マルソウダ *Auxis rochei*、マグロ属コシナガ *Thunnus tonggol*、マグロ属クロマグロ *T. orientalis*）の凍結精子を用いた人工授精を行い、生残性のスマ雑種を得ることが可能であるかを検討した。

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第 1 章

ゴマサバ×マサバ雑種の作出とその特性解析

Suitability of hybrid mackerel (*Scomber australasicus* × *S. japonicus*) with germ cell-less sterile gonads as a recipient for transplantation of bluefin tuna germ cells

Abstract

We aim to establish a small-bodied surrogate broodstock, such as mackerel, which produces functional bluefin tuna gametes by spermatogonial transplantation. When reproductively fertile e fish are used as recipients, endogenous gametogenesis outcompetes donor-derived gametogenesis, and recipient fish predominantly produce their gametes. In this study, we assessed fertility of hybrid mackerel, *Scomber australasicus* × *S. japonicus*, and its suitability as a recipient for transplantation of bluefin tuna germ cells. Hybrid mackerel were produced by artificially inseminating *S. australasicus* eggs with *S. japonicus* spermatozoa. Cellular DNA content and PCR analyses revealed that F1 offspring were diploid carrying both paternal and maternal genomes. Surprisingly, histological observations found no germ cells in hybrid mackerel gonads at 120 days post-hatch (dph), although they were present in the gonad of 30- and 60-dph hybrid mackerel. The frequency of germ cell-less fish was 100% at 120-dph, 63.1% at 1-year-old, and 81.8% at 2-year-old. We also confirmed a lack of expression of germ cell marker (DEAD-box helicase 4, *ddx4*) in the germ cell-less gonads of hybrid mackerel. By contrast, expression of Sertoli cell marker (gonadal soma-derived growth factor, *gsdf*) and of Leydig cell marker (steroid 11-beta-hydroxlase, *cyp11b1*) were clearly detected in hybrid mackerel gonads. Together these results showed that most of the hybrid gonads were germ cell-less sterile, but still possessed supporting cells and steroidogenic cells, both of which are indispensable for nursing donor-derived germ cells. To determine whether hybrid gonads could attract and incorporate donor bluefin tuna germ cells, testicular cells labeled with PKH26 fluorescent dye were intraperitoneally transplanted. Fluorescence observation of hybrid recipients at 14 days post-transplantation revealed that donor cells had been incorporated into the recipient's gonads. This suggests that hybrid mackerel show significant promise for use as a recipient to produce bluefin tuna gametes.

Introduction

Surrogate broodstock technology consists of producing donor-derived gametes in a surrogate fish by transplanting germline stem cells of a donor to a recipient of a different strain or species (Yoshizaki and Yazawa, 2019). Intraperitoneally transplanted donor germline stem cells migrate towards recipient genital ridges by chemotaxis and are eventually incorporated. The donor-derived germline stem cells start either spermatogenesis or oogenesis depending on the sex of the recipient fish (Yoshizaki and Lee, 2018). Using this technique, we successfully generated masu salmon (*Oncorhynchus masou*) that produce gametes of rainbow trout (*O. mykiss*) (Takeuchi et al., 2004; Okutsu et al., 2007; Lee et al., 2015; Yoshizaki et al., 2016). As an application of the surrogate broodstock technology in aquaculture, seed production of large-bodied and commercially important fish species would be possible in small land-based fish tanks using surrogate small-bodied parents with short generation times. Thus, we have attempted to establish a small-bodied surrogate recipient, such as mackerel, that produces functional bluefin tuna gametes. If realized, the strategy will save space, labor and cost for the seedling production of bluefin tuna. To date, surrogate recipients that produce allogeneic and xenogeneic gametes is established in various marine teleosts, including Carangidae (Morita et al., 2012, 2015), Tetraodontidae (Hamasaki et al., 2017, Yoshikawa et al., 2018b) and Sciaenidae (Yoshikawa et al., 2017, 2018a). We also developed a spermatogonial transplantation technique using chub mackerel as recipients (Yazawa et al., 2010, 2013).

To establish a surrogate broodstock for bluefin tuna, sterile mackerel recipients are desirable. When reproductively fertile fish are used as recipients, endogenous gametogenesis outcompetes donor-derived gametogenesis, resulting in dominant production of recipient gametes. We previously demonstrated that triploid recipients can produce only donor-derived gametes (Okutsu et al., 2007; Yoshizaki et al., 2010; Lee et al., 2013; Yoshikawa et

al., 2017; Hamasaki et al., 2017; Seki et al., 2017). As an option to triploidization, sterile recipients lacking endogenous germ cells caused by gene knockdown or knockout of the *dead end* (*dnd*) gene, which is required for the maintenance of primordial germ cells (Weidinger et al., 2003), has been used as recipients in several previous reports (Saito et al., 2008; Yoshizaki et al., 2016; Li et al., 2017; Octavera and Yoshizaki, 2019; Marinović et al., 2019).

However, application of these sterilization methods to marine fish is difficult due to their generally high mortality during larval development. Since the above-mentioned methods directly treat fertilized eggs, production of large numbers of sterile fish with is unrealistic with feeble marine fish larvae. Although triploidy in eastern little tuna (*Euthynnus affinis*) is successfully induced, triploid fish showed high mortality than the diploids during two to four weeks post-hatching because of selective cannibalism by diploid siblings (Yazawa et al., 2019). Thus, an alternative method suitable for marine fish is required for the mass-production of sterile recipient fish.

In this study, we focused on interspecific hybridization as an alternative approach. Hybrid sterility is a common phenomenon observed in many eukaryotic inter-species hybrids, including examples in yeast, plants, insects, birds, and mammals (Coyne and Orr, 2004; Maheshwari and Barbash, 2011). Interspecific hybridization is commonly used in the field of aquaculture to improve economic value. Typically, combinations of superior traits of parent species and positive heterosis are sought, and numerous examples of sterile hybrids are known for various fish species (Bartley et al., 2001; Rahman et al., 2013). Further, several reports that used sterile hybrids as recipients for germ cell transplantation (Yamaha et al., 2003; Shimada and Takeda, 2008; Wong et al., 2011; Xu et al., 2019). Recently, we found that hybrids of female blue dram (*Nibea mitsukurii*) × male white croaker (*Pennahia argentata*) display germ cell-less sterile gonads (Yoshikawa et al., 2018a). Importantly, these

hybrid recipients receiving blue dram spermatogonia, produced only donor-derived gametes (Yoshikawa et al., 2018a). These examples of use of hybrid recipients indicate that sterile hybrids can fully support gametogenesis of transplanted germ cells. Thus, interspecific hybridization can be an effective method to produce sterile recipients. Moreover, interspecific hybridization enables mass-production of recipient fish simply by *in vitro* insemination and requires no additional treatment of fertilized eggs.

We focused on blue mackerel (*Scomber australasicus*) and chub mackerel, belonging to Scombridae to produce sterile hybrids for the creation of broodstock for bluefin tuna. Since both mackerel species are distributed in the coastal waters of Japan, and their spawning areas and timing show a large overlap (Yukami et al., 2009), we expected that hybrid sterility may be the reason for reproductive isolation of these closely related species. In the present study, we produced hybrid mackerel using a combination of female blue and male chub mackerel and assessed gonadal development. Further, its suitability as surrogate broodstock to produce bluefin tuna gametes was evaluated.

Materials and methods

Spawning induction of broodstock

Blue mackerel and chub mackerel were caught in purse seines and held for a month in an offshore aquaculture net pen (144 m^2 × 5-m depth) located in Higashi-Matsuura, Saga prefecture, Japan. On December 2013, approximately 40 blue mackerel and 160 chub mackerel (400-600 g in body weight; 30-40 cm in fork length) were transferred to Tateyama Station (Banda), Field Science Center of the Tokyo University of Marine Science and Technology (34°97′N, 139°76′E). These broodstock fish were reared in a land-based 70-m^3 fiber reinforced plastic (FRP) circular tank with flow-through seawater (100 l/min) under a natural photoperiod until use. The broodstock were fed extruded pellets (White Ikusei no.4; Hayashikane Sangyo, Yamaguchi, Japan), defrosted sand eel, and krill to apparent satiety twice daily. On May 2014, 10 blue and chub mackerel were transferred from the 70-m^3 rearing tank to 5-m^3 FRP-tanks for spawning induction with flow-through seawater (25 l/min) at 20.5°C–21.2°C under a natural photoperiod. To induce final maturation of parental fish, gonadotrophin-releasing hormone agonist (GnRHa) for the spawning induction was administered by implantation of sustained-release cholesterol pellets (Amezawa et al., 2018). Cholesterol pellets containing GnRHa were prepared by a custom peptide synthesis service (Anygen, Jeollanam-do, Korea) and implanted into the abdominal cavity. Pellets contained GnRHa at a dose of 100 μg/kg BW.

Interspecific hybridization

Approximately 32 h after GnRHa treatment, the fish were anesthetized with 100 ppm. 2-phenoxyethanol (Fujifilm Wako Pure Chemical Co., Osaka, Japan). Ovulated eggs were obtained from female blue mackerel by gently squeezing its abdomen, and the eggs were collected into a 2,000 ml beaker. Approximately 1 ml of milt was collected from each chub

mackerel male (for hybrid) and each blue mackerel male (for control blue mackerel) by pressing its abdomen. Milt was kept on ice until use. To compare the early development of hybrid mackerel and control blue mackerel, blue mackerel eggs were fertilized with chub mackerel or blue mackerel sperm. Blue mackerel eggs were divided into two fractions each containing approximately 40,000 eggs. One fraction was mixed with 1 ml of chub mackerel sperm, and the other with 1ml blue mackerel sperm. Mixed eggs and sperm were subsequently activated by adding 1,000 ml natural seawater and rinsed twice with natural seawater. Eggs that rose to the water surface within 5 min after fertilization were defined as floating eggs and triplicate lots of 20 floating eggs were collected into 8 ml Petri dishes filled with sterile seawater and incubated at 20°C. The number of eggs that reached the two- to four-cell stages within 1–2 h of fertilization was recorded as fertilized egg count, and the number of larvae that hatched within 48 h of fertilization was recorded as hatched egg count. The fertilization rate was calculated as [fertilized egg count / 20 floating eggs × 100], and hatching rate calculated as [hatched larva count / 20 floating eggs × 100]. This cross experiment was repeated three times, using different batches of fertilized eggs.

Larval culture

Approximately 20,000 fertilized eggs of hybrid mackerel were transferred to a 100-l polycarbonate tank (440 mm diameter × 700 mm deep) with flow through seawater and maintained at 20°C–21°C with gentle aeration. Larval rearing was described previously (Yazawa et al. 2010). Briefly, feeding of larvae began 2 days post-hatch (dph). The rotifer *Brachionus rotundiformis*, fed with freshwater type of Chlorella (Super fresh Chlorella V12; Cholera Industry Co., LTD, Tokyo, Japan), was added to the tank twice a day. Densities of rotifers and *Nannochloropsis sp.* (Marine fresh; Marine-bio Inc., Kumamoto, Japan) in the tank were maintained at 10 individuals/ml and 5×10^5 cells/ml, respectively. *Artemia* nauplii

and an artificial diet (Otohime, Pacific Trading Co., Ltd., Fukuoka, Japan) were provided from 12- and from 14-dph, respectively. To increase the n-3 fatty acid concentration in live food, the rotifers and *Artemia* nauplii were incubated with Hyper Gloss (Nissin Marine Tech Co. Ltd., Kanagawa, Japan) for 6 to 12 h before feeding. Fish were reared in 100-l tanks for approximately 25 days, followed by transfer into 5-m^3 tanks. The photoperiod and water temperature were not modified from ambient conditions.

Polymerase chain reaction (PCR) and ploidy analysis.

Aneuploid, gynogenic, or androgenic offspring were previously produced by interspecific hybridization (Liu, 2010). Therefore, to confirm successful production of diploid hybrids, 10 hybrid larvae at 1-dph were subjected to DNA analyses against nuclear DNA (nDNA) or mitochondrial DNA (mtDNA). Detection of parental nDNA was performed using a multiplex PCR kit “Saba checker-I” (SCOTS, Saga, Japan) according to manufacturer's instructions. This method amplified both blue and chub mackerel specific regions in the nuclear ribosomal DNA (rDNA) internal transcribed spacer (ITS1) region. Identification of maternal origin was performed by PCR-restriction fragment length polymorphism (RFLP) analysis of mtDNA following a previously reported protocol (Food and Agricultural Materials Inspection Center and Fisheries Research Agency, 2007). Briefly, the PCR was performed with primer sets LSs1-Leu and HSs1-ND5 (Table. 1), designated for the conserved region located from tRNA-Leu (CUN) to NADH dehydrogenase subunit 5 (ND5) gene between chub and blue mackerel. PCR amplification was conducted in a 50-μl reaction volume containing 1× PCR Buffer II, 200 μM of dNTPs, 1.5mM $MgCl_2$, 1.25 U of Ampli*Taq* Gold DNA polymerase (Thermo Fisher Scientific, MA, USA), 50 ng of template DNA, and 1 μM of each primer. Thermal cycling conditions were: 1 cycle of 95°C for 8 min, then 35 cycles of 94°C for 30 s, 55°C for 15 s, 72°C for 1 min, followed by a final

elongation step at 72°C for 7 min. Amplified PCR fragments were digested for an hour at 37°C with *Hae* III, which recognizes a sequence unique to the target region of chub mackerel. PCR-RFLP products were electrophoresed on a 2.0% agarose gel. To estimate average cellular DNA contents of parental species and hybrid larvae at 1-dph, flow cytometric analysis was performed as described previously by Yazawa et al. (2019). Relative DNA content of each larva was measured using a Guava PCA-96 (Millipore, Billerica, MA). CyStain PI Absolute T kits (Partec, Munster, Germany) were used according to the manufacturer's instructions and a blue mackerel larva was used to represent the standard DNA content value of respective sample types. Flow cytometry was performed using ten 1-dph hybrid mackerel, control chub mackerel, and blue mackerel.

Histological analysis of hybrid mackerel gonads

Fish used in histological analyses were 30-, 60-, 120-dph, 1 and 2-year-old hybrid mackerel. Gonadosomatic indices (GSI; [gonad weight in grams/body weight in grams] × 100) were measured to monitor gonadal development of hybrid mackerel. Gonads were fixed with Bouin's fixative overnight at 4°C, cut into 4-μm thick sections using standard paraffin-embedding methods, and stained with hematoxylin and eosin. Images of sections were obtained using a light microscope (BX-51; Olympus, Tokyo, Japan) and a digital camera (DP-70; Olympus). This histological analysis was performed using at least 10 gonads of hybrid mackerel at each age.

Gene expression analyses of hybrid mackerel gonads

The localization of germ cell marker, DEAD-box polypeptide 4 (*ddx4*) mRNA, and Sertoli cell marker, gonadal soma derived growth factor (*gsdf*) mRNA, were analyzed by in situ hybridization (ISH) on tissue sections of 120-dph hybrid and blue mackerel. Antisense

RNA probes were synthesized from 379-bp chub mackerel *ddx4* (nucleotide 2,008-2,387 bp; GQ404693), and 488-bp chub mackerel *gsdf* (nucleotide 1-488 bp; GQ404694) cDNA fragments, as previously described (Yazawa et al., 2010). The ISH was performed as described previously (Sawatari et al., 2007). Since homologies of *ddx4* and *gsdf* probes between the two mackerel are 95.2% and 98.7%, respectively, these probes are expected to be hybridized to transcripts from alleles of both species. Total RNA extraction and cDNA synthesis were performed as previously described (Yazawa et al., 2010). To validate the histological observations, the reverse-transcription PCR (RT-PCR) for germ cell marker, *ddx4*; Sertoli cell marker, *gsdf*; Leydig cell marker, steroid 11-beta-hydroxylase (*cyp11b1*); and internal control, beta-actin (*actb*) was performed using cDNA obtained from the gonads of hybrid mackerel at 120-dph and 1-year-old. Moreover, to clarify whether hybrid mackerel have the potential to produce the 11-ketotestosterone (11-KT) that plays a pivotal role in spermatogenesis, RT-PCR for six steroidogenic enzyme genes required for conversion of cholesterol to 11-KT (cholesterol side-chain-cleavage enzyme, *cyp11a1*; 3 beta-hydroxysteroid dehydrogenase / delta 5-delta 4 isomerase type I, *hsd3b1*; steroid 17 alpha-hydroxylase / C17,20 lyase, *cyp17a1*; hydroxysteroid 17-beta dehydrogenase 12, *hsd17b12*; *cyp11b1* and hydroxysteroid 11-beta dehydrogenase 2, *hsd11b2*) were performed using cDNA obtained from the testis of hybrid mackerel at 1-year-old. The PCR amplification was conducted with AmpliTaq Gold DNA polymerase; primer sets for each gene are listed in Table 1. To detect transcripts from alleles of both species, all primers were designed against regions that are completely conserved between chub and blue mackerel. The GenBank accession number of each gene of both chub and blue mackerel is listed in Table 2. Thermal cycling conditions were: 1 cycle of 95°C for 10 min, then 35 cycles of 95°C for 30 s, 58°C for 30 s, and 72°C for 1 min, followed by a final elongation step at 72°C for 3 min. PCR products were electrophoresed on a 2.0% agarose gel.

Germ cell transplantation

Donor testicular cells were prepared from 3-year-old male Pacific bluefin tuna (body weight, approximately 40 kg) which were reared in net pens at Kushimoto, Wakayama Prefecture, Japan. Freshly isolated testes were minced with Weckel scissors and dissociated as previously described (Yazawa et al., 2013). To isolate spermatozoa and blood cells from whole testicular cell suspensions, density gradient centrifugation using a Percoll gradient (Percoll Plus; GE Healthcare, Princeton, NJ) was performed as previously described (Ichida et al., 2019). To label donor testicular cells, PKH26 (Sigma-Aldrich, Inc., St. Louis, MO) staining was performed as described by Takeuchi et al. (2009). We previously revealed that chub mackerel larvae with a total length of 5.3-mm showed higher incorporation efficiency of transplanted germ cells relative to those with a total length of 4.2-mm or 6.9-mm (Yazawa et al., 2010). In this study, therefore, PKH26-labeled cells were transplanted into the peritoneal cavity of hybrid mackerel larvae at 10-dph with a total length of 5.8-mm. At least 10,000 cells were injected into each of the 170 recipients. Transplantation was performed as previously described (Yazawa et al., 2010). Genital ridges excised from five recipients were imaged under a fluorescent microscope (BX51N-34FL, Olympus) at 14 days post-transplantation to confirm the incorporation of PKH26-labeled donor germ cells. Incorporation rate of donor-derived germ cells in recipient genital ridges was calculated as [number of fish incorporating fluorescent cells in genital ridges at 14 days post-transplantation/number of fish observed x 100].

Statistical analysis

All data are represented as the mean ± standard error of the mean (SEM). A value of $p < 0.01$ was considered significant for all tests. A two-tailed Student's t-test was used to

determine statistical differences in means of fertilization rates and hatching rates between hybrid mackerel and chub mackerel (F-test was performed to show that the variance of populations were equal). Further, one-way analysis of variance (ANOVA), followed by Tukey's multiple comparison test was used to determine statistical significance in the mean GSI of 1- and 2-year-old hybrid mackerels and 1-year-old chub mackerels. Before conducting the ANOVA, the homogeneity of variances was determined with Bartlett's test. All analyses were carried out using GraphPad Prism version 5.0 (GraphPad).

Results

Production of hybrid mackerel

Early survival of hybrid mackerel is illustrated in Fig. 1A. Mean ± SEM values for fertilization and hatching rates of hybrid mackerel were 93.5% ± 2.3% and 87.4% ± 4.4% (n = 3), respectively. By comparison, mean fertilization and hatching rates of control blue mackerel were 96.7% ± 0.6% and 84.7% ± 3.6% (n = 3), respectively. No significant differences between these fertilization and hatching rates were found. Genomic DNA of hybrid mackerel larvae at 1-dph was subjected to multiplex PCR analysis that targeted a species-specific sequence of the ITS region in nDNA (Fig. 1B). As a result, both blue mackerel and chub mackerel specific fragments (200 bp and 125 bp, respectively) were amplified in all tested hybrid mackerel larvae (n = 10). Moreover, the PCR-RFLP targeted region of mtDNA indicated that hybrid mackerel larvae possessed mtDNA derived from blue mackerel; restriction patterns were identical to those of blue mackerel (Fig. 1C, n=10). Analyses of cellular DNA content revealed that hybrid mackerel showed the same peaks for DNA contents observed in diploid parental species and none exhibited signs of aneuploidy (Fig. 1D). Moreover, the external appearance of hybrid mackerel at 120dph was similar to parental species at the same age and showed no deformation. Hybrid fish grew normally (Fig. 1E).

Gonadal development of hybrid mackerel at 30-, 60- and 120-dph

To clarify the fertility of hybrid fish, gonadal development was compared with that of control chub mackerel (Fig. 2). At 30-dph, hybrid mackerel possessed sexually undifferentiated gonads with primordial germ cells (PGCs) that were enclosed by gonadal somatic cells (Fig. 2B), as also observed in controls (Fig. 2A). Control chub mackerel gonads at 60-dph differentiated into testis or ovary (Fig. 2C, D) and sperm duct and spermatogonia

were observed in testis (Fig. 2C). The ovarian cavity and ovarian lamella were formed in control fish, and oogonia were observed (Fig. 2D). By contrast, all hybrid mackerel at 60-dph showed only testis-like gonads possessing sperm duct and spermatogonia (n = 10, Fig. 2E). In control at 120-dph, numbers of germ cells were drastically increased in both testis (Fig. 2F) and ovary (Fig. 2G). In ovaries, primary oocytes were observed during the perinucleolus stage (Fig. 2G). Hybrid mackerel at 120-dph showed only testis-like gonads as at 60-dph, and germ cells were not observed in these testis-like gonads (n = 10, Fig. 2H).

Gene expression analyses of germ cell-less gonads of hybrid mackerel at 120-dph

To further confirm depletion of germ cells, we investigated expression of the germ cell marker, *ddx4* mRNA, in hybrid mackerel gonads at 120-dph by ISH (Fig. 3A-F, A'-F'). *ddx4*-positive germ cells were detected in control blue mackerel testis (Fig. 3B, B'), whereas no *ddx4*-positive germ cells were detected in hybrid mackerel gonads (Fig. 3E, E'). Further, transcripts for *ddx4* were not detectable using RT-PCR (Fig. 3G). *gsdf*-positive Sertoli cells were detected in both control blue mackerel and hybrid mackerel at nearly equal levels (Fig. 3C, C', F, F'). Thus, hybrid mackerel are devoid of germ cells, but not Sertoli cells. Expression of *gsdf* in the hybrid gonads was further confirmed by RT-PCR (Fig. 3G), as was the of Leydig cell marker, *cyp11b1* (Fig. 3G). These results are promising for germ cell transplantation because the presence of supporting cells and steroidogenic cells that can nurse donor-derived germ cells is a prerequisite for the successful colonization and development of these cells.

Histological analysis of hybrid mackerel gonads at 1- and 2-year-old

Male and female chub mackerel are reported to reach sexual maturity at one year in captivity (Ishibashi et al., 2007; Nyuji et al., 2014). We investigated 1- and 2-year-old hybrid

mackerel for germ cell-less testis during spawning seasons. At one and two years after hatching, seven out of 11 (63.6 %) and eight out of 11 (72.7 %) hybrid mackerel possessed germ cell-less testes, respectively (Fig. 4A, G, M) and GSI of these germ cell-less testes was 0.04 ± 0.003% and 0.04 ± 0.007%, respectively both of which are significantly lower than testes of control chub mackerel at 1 year old (GSI; 6.75 ± 0.758% , Fig. 4E, K, Q) ($p > 0.01$). Three out of 11 (27.3%) 1-year-old and 2 out of 11 (18.2%) 2-year-old hybrid mackerel possessed spermiated testes and all stages of spermatogenic cells, i.e. spermatogonia to sperm, was observed with HE staining (Fig. 4C, I, O), yet the GSI of these spermiated testes was 0.31 ± 0.12% and 1.9 ± 1.3%, respectively, were also significantly lower than testes of 1 year-old control chub mackerel ($p > 0.01$). Also, one out of 11 (9.1%) 1-year-old hybrid mackerel showed displayed an ovary with numerous oocytes (Fig. 4D, J, P). Moreover, one out of 11 (9.1%) 2-year-old hybrid mackerel possessed germ cell-less ovary-like gonad with an ovarian cavity and ovarian lamella (Fig. 4B, H, N). As mentioned above, although a 50:50 sex ratio is observed in wild chub mackerel, the sex ratio of male to female hybrid mackerel was 10:1 at both 1- and 2-years after hatching (Table 3). Thus, hybrid mackerel exhibit a strong male-biased sex ratio.

Gene expression analyses of testis of hybrid mackerel at 1-year-old

The expression of *ddx4*, *gsdf* and six steroidogenic enzyme genes required for conversion of cholesterol to 11-KT (*cyp11a1*, *hsd3b1*, *cyp17a1*, *hsd17b12*, *cyp11b1*, *hsd11b2*) in the testis of 1-year-old hybrid mackerel were investigated using RT-PCR (Fig. 5). The germ cell-less testis of 1-year-old hybrid mackerel were found to be *ddx4*-negative, *gsdf*-positive, and *cyp11b1*-positive. Thus, the expression pattern of each cell-type maker genes in the germ cell-less testis of 1-year-old fish was the same as those at 120-dph. Moreover, all tested steroidogenic enzyme genes were detected in both germ cell-less testis (*ddx4*-negative) and

spermiated testis (*ddx4*-positive) of hybrid mackerel, as well as in the spermiated testis of control blue and chub mackerel. These results suggested that the germ cell-less testis of hybrid mackerel have the ability to produce the 11-KT.

Transplantation of bluefin tuna testicular cells into the hybrid mackerel.

Bluefin tuna testicular cells labeled with PKH26 (Fig. 6A, B) were transplanted into the peritoneal cavity of hybrid larvae at 10-dph. At 24-dph, no fluorescence was observed in the gonads of non-transplanted fish (Fig. 6C-E), whereas numerous PKH26-labeled cells were incorporated in the gonad of transplanted recipient larvae (Fig. 6F-H). The frequency of occurrence of recipient larvae incorporating donor-derived bluefin tuna germ cells was 100% (n = 5). The mean number of donor-derived germ cells found in the hybrid mackerel recipients was 36.9 ± 8.4. Hybrid mackerel gonads thus possessed a capability to attract and incorporate donor germ cells of bluefin tuna at high efficiency.

Discussion

In this study, we succeeded in producing a hybrid mackerel (female blue mackerel × male chub mackerel) that lost germ cells in gonads after 120-dph. This loss was confirmed by lack of expression of a germ cell marker, *ddx4.* In contrast, gene expression of a Sertoli cell marker, *gsdf*, and a Leydig cell marker, *cyp11b1*, were clearly detected in the hybrid mackerel gonads. The hybrid mackerel thus shows germ cell-less sterility but retains supporting cells and steroidogenic cells both of which are essential for nursing transplanted exogenous germ cells. More importantly, bluefin tuna germ cells transplanted into the peritoneal cavity migrated to and were incorporated into hybrid mackerel gonads with high efficiency. The genital ridge of hybrid mackerel thus retains the capability to attract and incorporate xenogeneic bluefin tuna germ cells.

As a key regulator for guiding the migration of PGCs, the chemokine SDF-1 (stromal cell-derived factor 1, also called as CXCL12) secreted by the region where the gonad develops and its receptor CXCR4 (C-X-C chemokine receptor type 4) expressed in the PGCs have been identified in zebrafish (Doitsidou et al., 2002), medaka (Sasado et al., 2008), and kingfish (Fernández et al., 2015). We previously confirmed CXCR4 is also expressed in testicular germ cells (Hayashi et al., unpublished data). Therefore, hybrid mackerel SDF-1 may activate bluefin tuna CXCR4 signaling and guide the migration of transplanted bluefin tuna germ cells to the region where the gonad develops, even though they were isolated from a different genus. In a previous study, migration of donor cells into recipient gonads was usually possible even if donor and recipient were genetically distant species (Yoshizaki and Yazawa, 2019; Goto and Saito, 2019). Notably, Saito et al. (2014) showed that transplanted sturgeon PGC was incorporated into the genital ridge of goldfish. These species are from different orders. These reports suggest that mechanisms of germ cell migration are conserved across fish species, and the present results are consistent with this hypothesis.

Incorporation rate (100.0%) found in the present inter-generic transplantation was higher than colonization rates (70.0%) observed in our previous inter-family transplantation using donor nibe croaker and recipient chub mackerel (Yazawa et al., 2010). Colonization frequency may be affected by the phylogenic distance between the donor and recipient species.

Overall, hybrid mackerel show significant promise as a recipient for production of bluefin tuna gametes by surrogate broodstock technology. Most hybrid mackerel are germ cell-less and hold advantages over sterile triploid fish. First, germ cell-less recipients produce only donor-derived gametes. Triploids produce small numbers of abnormal, aneuploid gametes in some species (Felip et al., 2001; Piferrer et al., 2009), leading to risks of producing both donor-derived gametes and dysfunctional endogenous gametes. Consequently, the efficiency of producing donor-derived offspring might be compromised undesirable fertilization with dysfunctional endogenous gametes. Since germ cell-less recipients are completely devoid of endogenous germ cells, they will produce only donor-derived offspring. Second, since triploid gonads maintain a normal number of mitotic germ cells, endogenous cells are presumed to compete with the transplanted germ cells for germ cell niches. No such competitive endogenous germ cells exist in germ cell-less sterile fish. In rainbow trout, the donor-derived germ cells showed significantly higher colonization and proliferation efficiency in the gonads of germ cell-less recipients, produced by knockdown of *dnd* expression using antisense morpholino oligonucleotides (AMO), than those in the gonads of the control recipients (Yoshizaki et al., 2016). In contrast to *dnd*-AMO-treated recipients that show no germ cells during the transplantation period, hybrid mackerel possess endogenous germ cells until 60-dph. Therefore, the colonization efficiency of donor-derived germ cells might not be enhanced in the gonads of hybrid mackerel. On the other hand, since endogenous germ cells of most hybrid mackerels disappeared by 120-dph, the transplanted

germ cells can occupy the germ cell niches effectively, meaning that their proliferation would be enhanced in the gonads of hybrid mackerel. Further, when testicular cells of sockeye salmon (*Oncorhynchus nerka*) are transplanted into triploid and *dnd*-knockdown rainbow trout, triploid trout produces donor-derived salmon sperm but not eggs, but the *dnd*-knockdown trout recipient successfully produces both donor-derived salmon sperms and eggs (Yoshizaki et al., unpublished data). In the present study, most hybrid mackerel possess germ cell-less gonads, making them promising recipients for production of xenogeneic bluefin tuna gametes.

In various animal species, including fish, a cytological mechanism of hybrid sterility is meiotic arrest caused by failure to achieve synapsis between homologous chromosomes (Chandley et al., 1975; Shimizu et al., 1997; Sawamura et al., 2004; Bhattacharyya et al., 2013; Islam et al., 2013). Therefore, germ cell-less sterility in the hybrid mackerel is a rare case of hybrid sterility. Recently, Yoshikawa et al. (2018a) reported that a hybrid of female blue dram × male white croaker showed germ cell-less sterility, and this report is apparently to only one in the existing literature. The PGCs of hybrid larvae from this cross migrate and are incorporated into genital ridges normally but are unable to proliferate. Eventually, hybrid gonads become germ cell-less and sterile. In contrast, proliferation of PGCs in mackerel hybrid gonads was observed histologically at 60-dph, indicating that germ cell-less sterility of hybrid mackerel is caused by mechanisms different from mechanisms underlying sterility in blue dram × white croaker hybrids. Further study is required to investigate proliferation, differentiation, and apoptosis in germ cells found in the gonads of hybrid mackerel juveniles.

We revealed that hybrid mackerel show a strong male-biased sex ratio and individuals possessing ovary-like germ cell-less gonads rarely appeared. In zebrafish and medaka (*Oryzias latipes*), numbers of germ cells affect sex differentiation; that is, absence of germ cells leads to exclusive male development even in genetic females (Weidinger et al., 2003;

Slanchev et al., 2005; Kurokawa et al., 2007; Li et al., 2017). This phenomenon also occurred in hybrid mackerel possessing germ cell-less gonads. This male-biased sex ratio may become a potential disadvantage for production of donor-derived eggs. However, we have succeeded in producing feminized chub mackerel by oral administration of estradiol-17beta (Tani et al., unpublished data), the promising potential for production of female hybrid mackerel using this technique will be assessed in future work.

In order to nurse the spermatogenesis of transplanted exogenous germ cells, it is essential that the recipient has the ability to produce the androgens (11-KT) that play a pivotal role in spermatogenesis (Miura et al., 1991, Schulz and Miura, 2002). The RT-PCR analysis revealed that the steroidogenic enzyme genes, required for conversion of cholesterol to 11-KT, are expressed in the germ cell-less testis of 1-year-old hybrid mackerel. Since the germ cells depletion markedly affects the anatomical structure and frequency of occurrence of each cell type in testes, quantitative analyses of reproductive hormones in the germ cell-less testis were not performed. Certainly, however, the germ cell-less testis of hybrid mackerel do have the potential for androgen production. Future studies will be required to reveal whether transplanted hybrid mackerel recipients can nurse spermatogenesis of donor-derived germ cells.

The sterility of hybrid mackerel was not displayed in all individuals. The frequency of non-sterile fish was as low as 36.4% after 1 year, and 18.2% after 2 years, and 0% after the first four months (120-dph group). Since we produced these fish groups using different broodstock, we could not rule out the possibility that the frequency of the sterile fish is affected by their genetic background. To produce a 100% sterile population, the precise mechanism of sterility caused by hybridization in mackerel will require better understanding.

In the present study, we showed biological characteristics of hybrid mackerel, with special emphasis on germ cell-less sterility. We also showed applicability of this hybrid for

mass-production and its ability to harbor tuna germ cells. Thus, hybrid mackerel are promising recipient candidates as surrogate broodstock for bluefin tuna gamete production.

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Figure legends

Figure 1. Production of hybrid mackerel.

A: Fertilization and hatching rates of blue mackerel (BM) eggs inseminated with BM sperm and chub mackerel (CM) sperm. Cross experiments were performed at three times and data are presented as mean ± SEM. **B**: Species-specific PCR amplification of nuclear DNA region in hybrid mackerel larvae. Lanes 1 - 10 are genomic DNA obtained from hybrid mackerel larvae at 1-dph. Lanes CM and BM are positive controls obtained using genomic DNA extracted from chub mackerel and blue mackerel, respectively. Lane CM+BM is a positive control obtained by mixing genomic DNA of chub mackerel and blue mackerel. **C**: PCR-RFLP analysis of the mitochondrial DNA region in hybrid mackerel larvae. Amplified PCR fragments were undigested (U) and digested (D) with the restriction enzyme *Hae* III, which recognizes species-specific sequence of chub mackerel DNA. Lanes 1 - 4 (corresponding to lanes 1-4 in Fig.1B) are genomic DNA obtained from hybrid mackerel larvae at 1-dph. Lanes CM and BM are positive controls obtained using genomic DNA extracted from chub mackerel and blue mackerel, respectively. **D**: Representative histogram showing the relative nuclear DNA contents of chub mackerel (CM), blue mackerel (BM), and hybrid mackerel (BM × CM hybrid). **E**. Typical external appearance at 120 dph of chub mackerel (CM), blue mackerel (BM) and hybrid mackerel (BM×CM hybrid). Bars = 2 cm.

Figure 2. Gonadal development of chub mackerel and hybrid mackerel at 30, 60 and 120-dph.

Hematoxylin and eosin-stained transverse sections of chub mackerel gonads (A, C, D, F, G) and hybrid mackerel gonads (B, E, H). Arrowheads indicate germ cells. **A-B**: At 30-dph, sexually undifferentiated gonads with a primordial germ cell were observed in chub

mackerel (A) and hybrid mackerel (B). **C-E**: SD, sperm duct; OC, ovarian cavity. At 60-dph, sexually differentiate testis with spermatogonia (C) and ovary with oogonia (D) were observed in chub mackerel, whereas only testis-like gonads with spermatogonia were observed in hybrid mackerel (E). **F-H**: At 120-dph, testis with proliferated spermatogonia (F) and ovary with oocytes (G) were observed in chub mackerel, whereas only testis-like gonads without germ cells were observed in hybrid mackerel (H). Bars = 20 μm.

Figure 3. Gene expression analyses in germ cell-less gonads of hybrid mackerel at 120-dph **A-F**, **A'-F'**: *in situ* hybridization analysis of *ddx4* and *gsdf* gene expression in germ cell-less gonad of hybrid mackerel and testis of blue mackerel. Sequential sections of blue mackerel testis (A-C, A'-C') and hybrid mackerel (D-F, D'-F') are stained with Hematoxylin and eosin (left columns; A, A', D, D') and hybridized with *ddx4* (middle columns; B, B', E-E') and *gsdf* (right columns; C, C', F, F') probes. Yellow broken lines indicate the cyst structure and red broken lines indicate the germ cells. Higher magnification views of the boxed area in A-F are shown in A'-F', respectively. Bars = 50 μm (A-F) and 20 μm (A'-F'). **E**: RT-PCR analysis of germ cell and gonadal somatic cell-specific genes in germ cell-less gonads of hybrid mackerel (BM×CM hybrid) and the testis and ovary of blue mackerel (BM) and chub mackerel (CM). RT-PCR was performed with primer sets specific to a germ cell marker (*ddx4*), Sertoli cell-marker (*gsdf*) and Leydig cell marker (*cyp11b1*). beta-actin (*actb*) is used as an internal control. N.C. represents a negative control with distilled water instead of template cDNA.

Figure 4. Typical gonad type observed in 1- and 2-year-old hybrid mackerel and 1-year-old chub mackerel in spawning season.

From top to bottom of columns, respectively: a germ cell-less testis of 2-year-old hybrid mackerel (**A,G,M**), a germ cell-less ovary of 2-year-old hybrid mackerel (**B,H,N**), testis of 1-year-old hybrid mackerel (**C,I,O**), ovary of 1-year-old hybrid mackerel (**D,J,P**), testis of control chub mackerel (**E,K,Q**), ovary of control chub mackerel (**F,L,R**) of external feature (left columns, A-F) and transverse section stained with Hematoxylin and eosin (middle columns, G-L). M-R are high magnification images of G-L, respectively (right columns). Bars = 2 cm (A-F), 100 μm (F-L) and 20 μm (M-R).

Figure 5. RT-PCR analysis of steroidogenic enzyme genes in the testis of 1-year-old hybrid mackerel (BM×CM hybrid), blue mackerel (BM♂), and chub mackerel (CM♂). Lanes 1-3 are cDNA obtained from spermiated testes of hybrid mackerels and lanes 4-10 are cDNA obtained from germ cell-less testes of hybrid mackerels. RT-PCR was performed with primer sets specific to a germ cell marker (*ddx4*), Sertoli cell-marker (*gsdf*) and steroidogenic enzyme genes required for conversion of cholesterol to 11-KT (*cyp11a1*, *hsd3b1*, *cyp17a1*, *hsd17b12*, *cyp11b1*, *hsd11b2*). The marker, *actb*, was used as an internal control. N.C. represents a negative control with distilled water instead of template cDNA.

Figure 6. Incorporation of transplanted xenogeneic bluefin tuna germ cells into gonads of hybrid mackerel recipient.

A-B: Dissociated bluefin tuna testicular cells labeled with red fluorescent dye (PKH26). All cells in the brightfield view (A) are strongly stained in the fluorescent view (B). **C-H**: Bright field (C, F) and fluorescent (D, E, G, H) views of excised gonads of non-transplanted (C-E) and transplanted (F-H) hybrid mackerel. The dotted lines indicate the gonads of hybrid mackerel. E and H are higher magnification of red rectangles in D and G, respectively. Bars = 20 μm.

Fig. 1

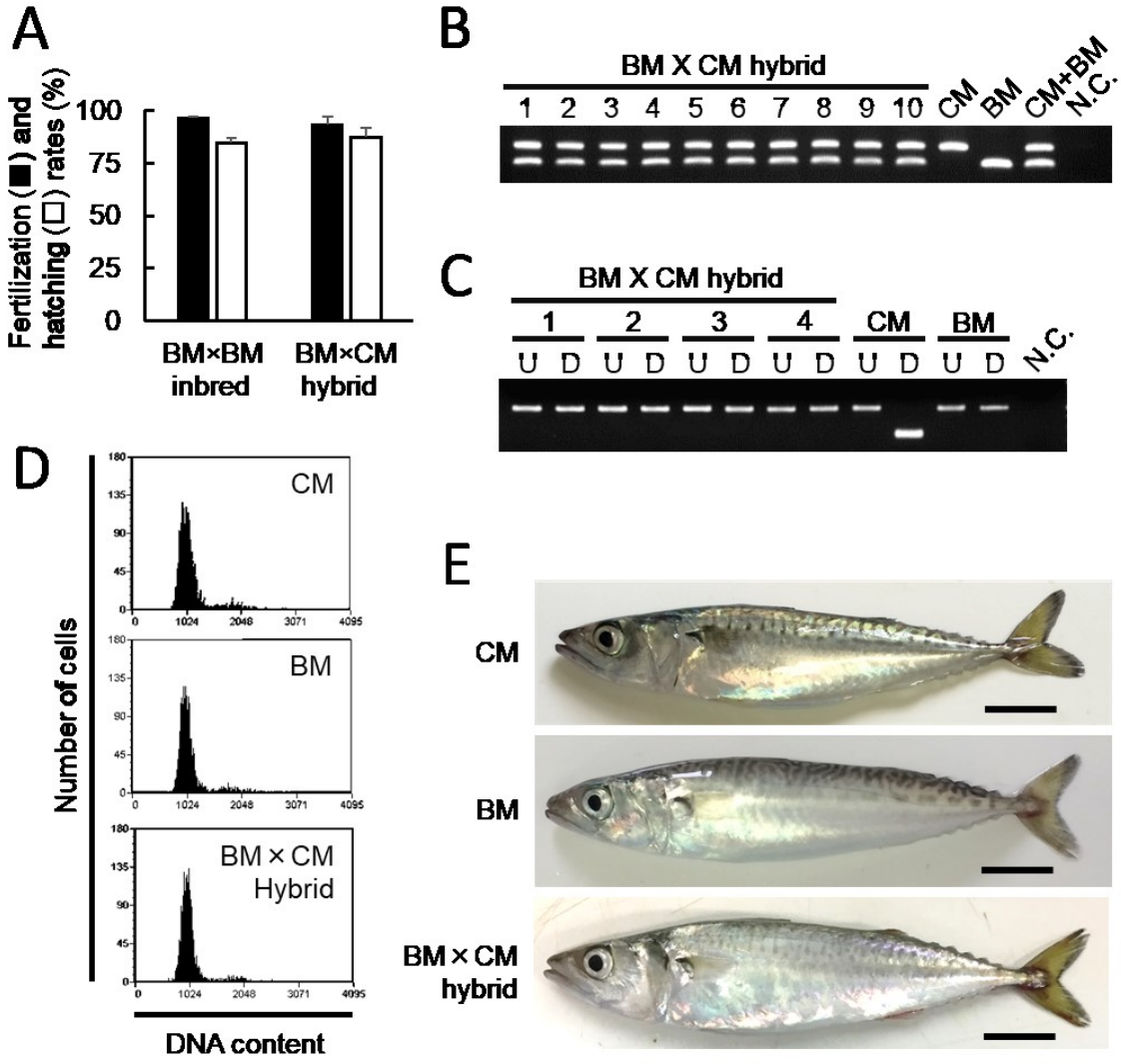
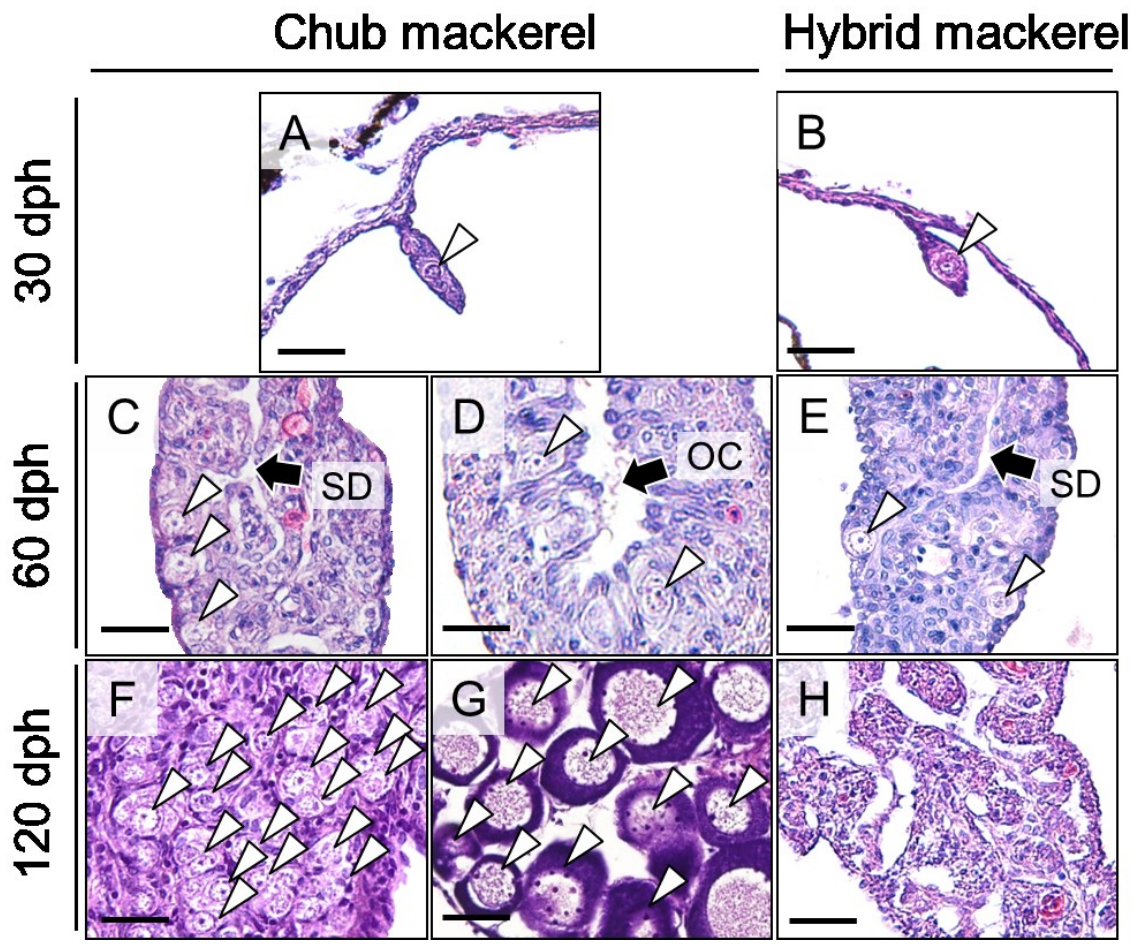
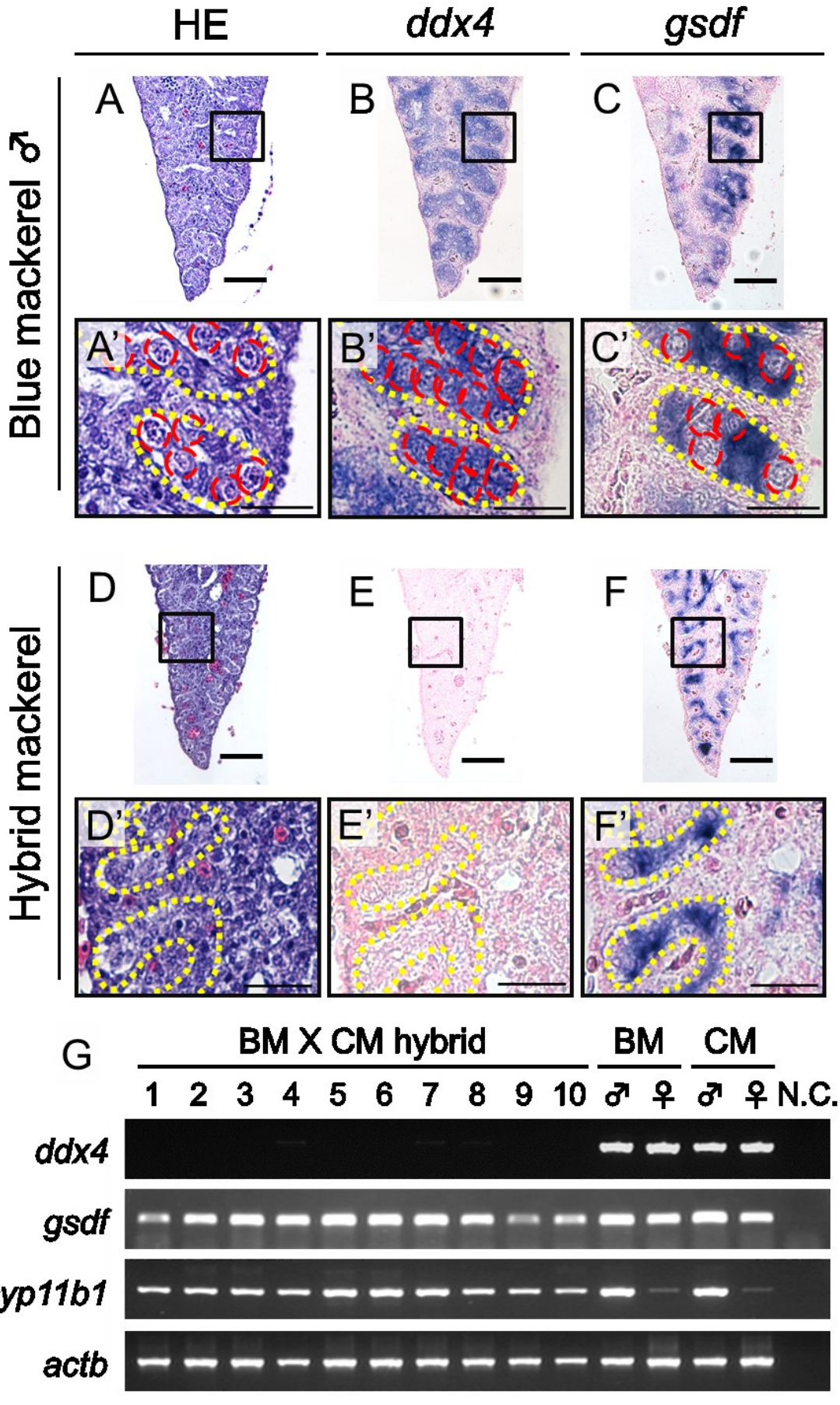

A
Fertilization (■) and hatching (□) rates (%)
100
75
50
25
0
BM×BM inbred
BM×CM hybrid
B
BM X CM hybrid
1 2 3 4 5 6 7 8 9 10
CM
BM
CM+BM
N.C.
C
BM X CM hybrid
1 2 3 4
CM
BM
U D U D U D U D U D U D
N.C.
D
Number of cells
CM
BM
BM × CM Hybrid
0 1024 2048 3071 4095
DNA content
E
CM
BM
BM × CM hybrid

Fig. 2


Chub mackerel
Hybrid mackerel
30 dph
60 dph
120 dph
A
B
C
D
E
F
G
H
SD
OC
SD

I


HE
ddx4
gsdf
Blue mackerel ♂
A
B
C
A'
B'
C'
Hybrid mackerel
D
E
F
D'
E'
F'
G
BM X CM hybrid
BM
CM
1 2 3 4 5 6 7 8 9 10 ♂ ♀ ♂ ♀ N.C.
ddx4
gsdf
cyp11b1
actb

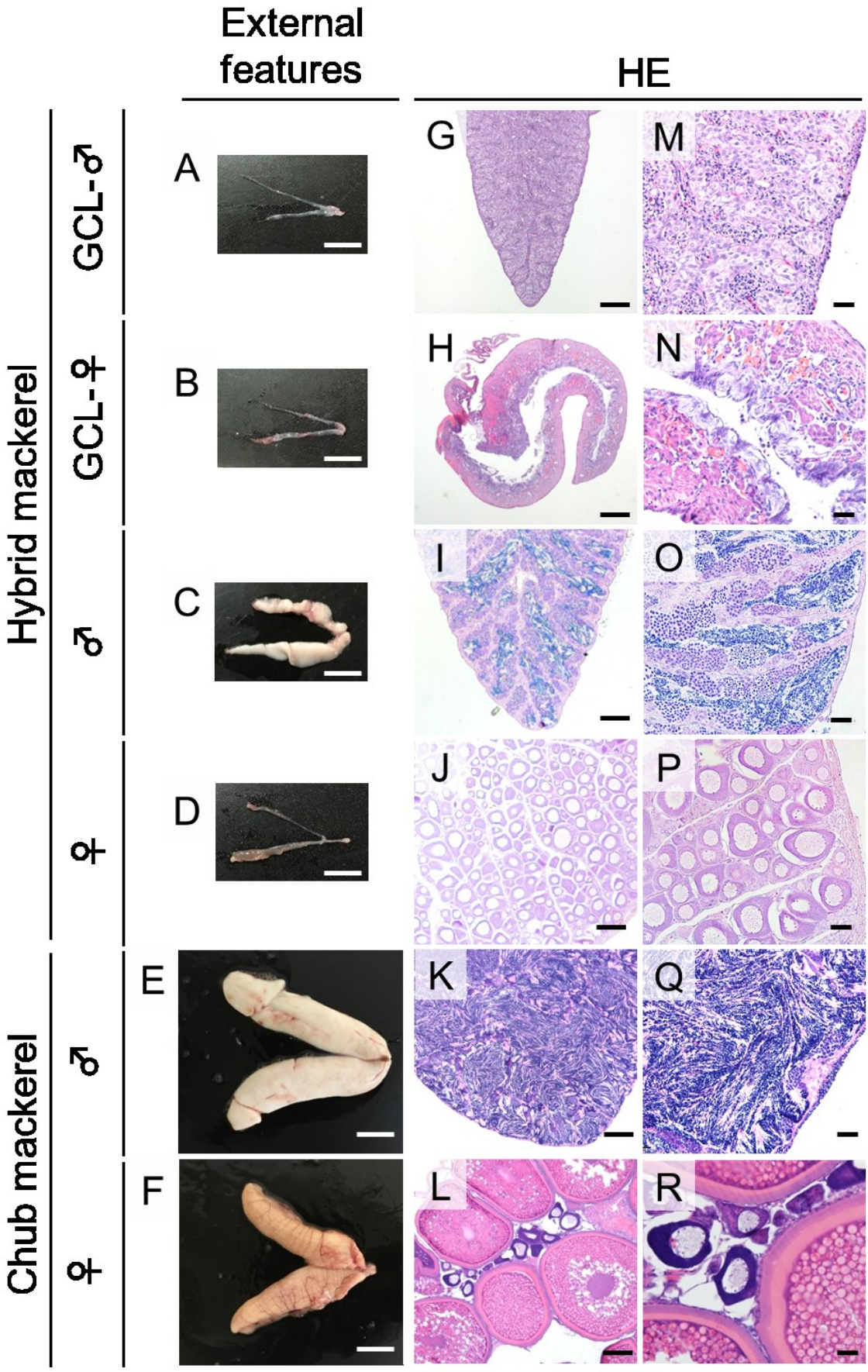

External features
HE
Hybrid mackerel
GCL-♂
GCL-♀
♂
♀
Chub mackerel
♂
♀
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P
Q
R

Fig. 5

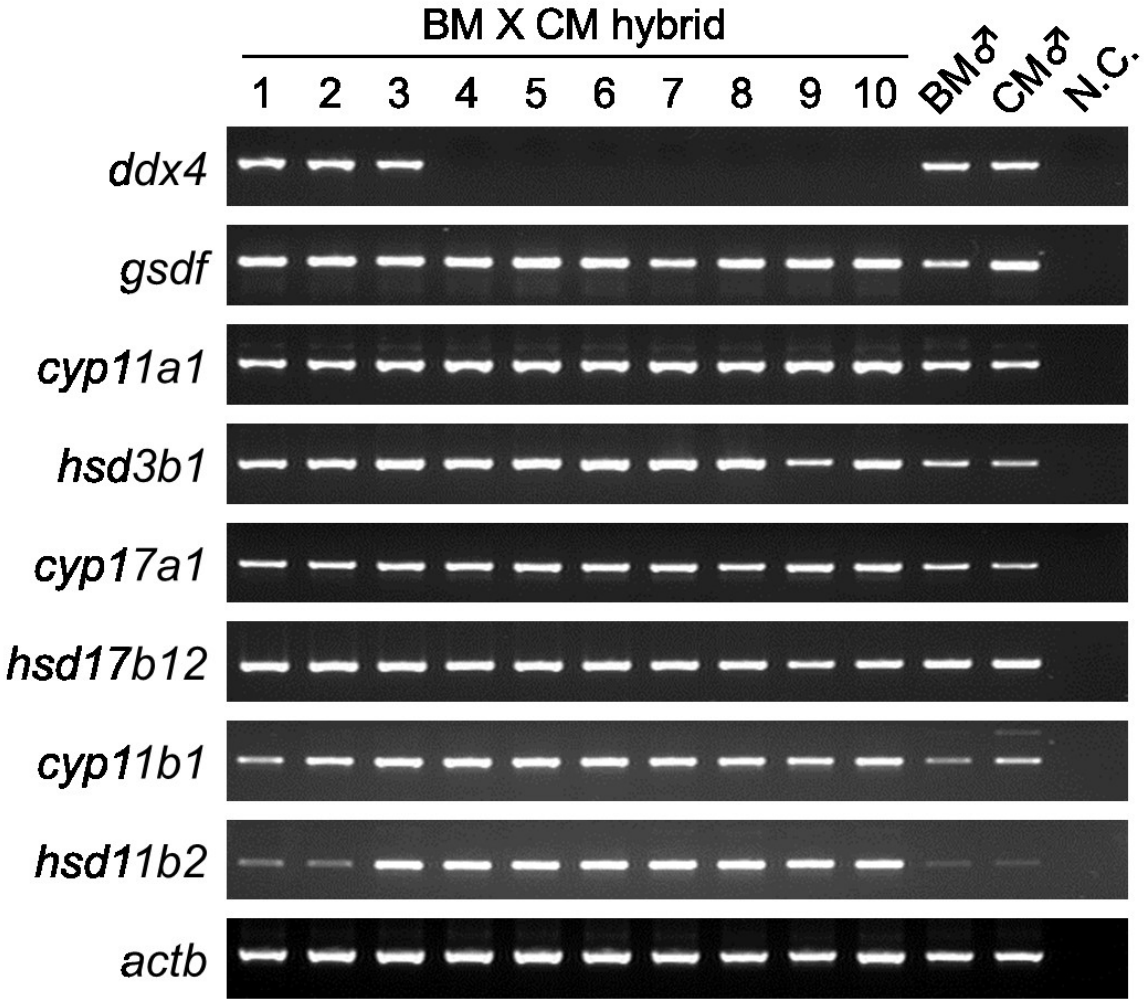

BM X CM hybrid
1
2
3
4
5
6
7
8
9
10
BM♂
CM♂
N.C.
ddx4
gsdf
cyp11a1
hsd3b1
cyp17a1
hsd17b12
cyp11b1
hsd11b2
actb

Fig. 6

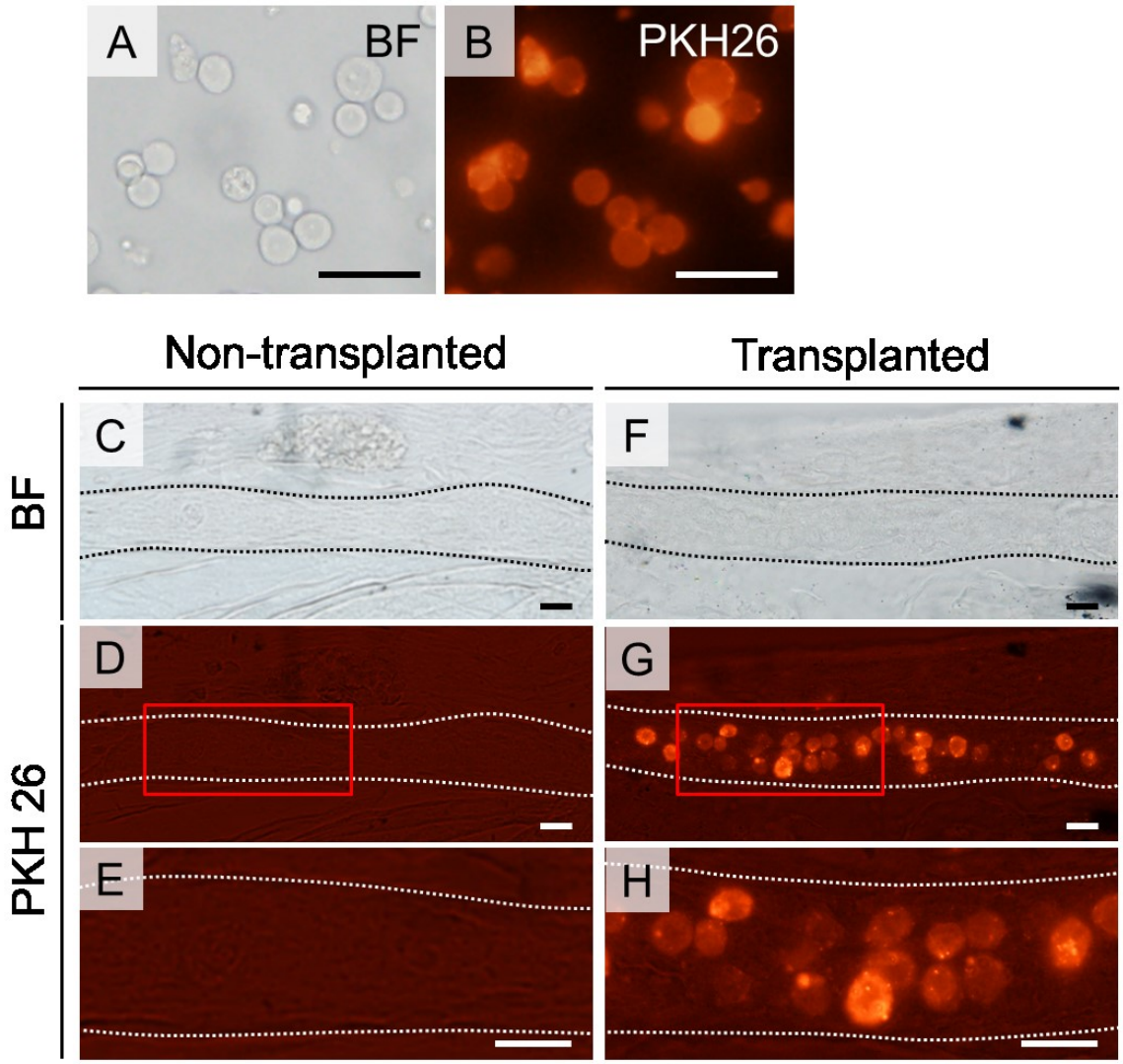


Table 1. List of primers

gene	Primer name	Primer sequence
tRNA-Leu (CUN)	LSs1-Leu	5'- ATCCGCTGGTCTTAGGAACC
ND5	HSs1-ND5	5'- CCTTCTCAGCCGATAAATAGTT
ddx4 (vasa)	Scomb_common_vasa_Fw	5'- CTATTTGTTCCTGGCTGTGG
	Scomb_common_vasa_Rv	5'- GCAGACTCTTCTAACCATGAAGG
gsdf	SC_gsdf_F1	5'- GCTCTCAACTTGCAGGCTGA
	SC_gsdf_R1	5'- CCCAGCCCAGATCTTTCATG
cyp11a1	Scomb_cyp11a1_Fw	5'- GGATTGGAGCAAAAGTGT
	Scomb_cyp11a1_Rv	5'- CTGGAATGTGGTAGTTTTGA
hsd3b1	Scomb_hsd3b1_Fw	5'- ACACAGCTGCTCCTAGAA
	Scomb_hsd3b1_Rv	5'- TCACGGGTGGTGTATCAT
cyp17a1	Scomb_cyp17a1_Fw	5'- TCTCTGTGTTCCATCCTCT
	Scomb_cyp17a1_Rv	5'- CCCCCCCAATTTACTGTC
hsd17b12	Scomb_hsd17b12_Fw	5'- CCATCCTCAACATCTCATC
	Scomb_hsd17b12_Rv	5'- CAAACCCATCCCCATCAC
cyp11b1	Scomb_cyp11b1_Fw	5'- CTGGGTCAATCTGGTGAAATT
	Scomb_cyp11b1_Rv	5'- CGCAGCATTCGAGAGAAAT
hsd11b2	Scomb_hsd11b2_Fw	5'- CATCCTACCGTCCTCCTA
	Scomb_hsd11b2_Rv	5'- CATCAGCTTCTTCTTCACA
actb	Scomb_actin_Fw	5'- ATGGTTGGTATGGGCCAGAA
	Scomb_actin_Rv	5'- GCTTCTCCTTAATGTCACGC

Table 2. List of GenBank accession numbers

gene	GenBank accession number	
	Chub mackerel *Scomber japonicus*	Blue mackerel *Scomber australasicus*
ddx4 (*vasa*)	GQ404693	GU581279
gsdf	GQ404694	MT470269
cyp11a1	MT459650	MT459651
hsd3b1	MT459657	MT459658
cyp17a1	MT459659	MT459660
hsd17b12	MT459654	MT459655
cyp11b1	MT459648	MT459649
hsd11b2	MT459653	MT459652
actb	GU731674	MT459656

Table 3. Occurrence frequency of gonad types and associated gonad somatic index (GSI)

	Hybrid mackerel				Chub mackerel (control)	
	1 year old		2 years old		1 year old	
Gonad types	Number of fish	GSI (%)	Number of fish	GSI (%)	Number of fish	GSI (%)
GCL testis*	7	0.04 ± 0.003 [a]	8	0.04 ± 0.007 [a]	0	
GCL ovary*	0		1	0.01	0	
Testis	3	0.31 ± 0.116 [a]	2	1.90 ± 1.325 [a]	10	6.75 ± 0.758 [b]
Ovary	1	0.1	0		8	6.62 ± 0.675 [b]
Total	11		11		18	

*GCL, germ cell-less.

Different letters indicate statistically significant differences (Tukey's test. $p < 0.01$).

第 2 章

サバ科魚類における野外での簡便な精子凍結法の開発

Development of a simple method for sperm cryopreservation of Scombridae fishes in outdoor environments

Abstract

We developed a simple dry-shipper method for cryopreserving the sperm of Scombridae fishes in outdoor environments. First, we undertook a preliminary study to optimize the sperm cryopreservation conditions using bullet tuna, *Auxis rochei* (Risso, 1810) sperm. We found that the optimum cryomedium contained 90% fetal bovine serum (FBS) or 300 mM trehalose as an external cryoprotectant and 10% dimethyl sulfoxide (DMSO) as an internal cryoprotectant. Under these optimized conditions, the post-thaw sperm had a duration of motility of 500 s and a motility rate of >70%. We then performed practical trials of the optimized protocol in various outdoor environments (e.g, fishing boats and ports) using the sperm of five Scombridae species: chub mackerel, *Scomber japonicus* (Houttuyn, 1782); blue mackerel, *S. australasicus* (Cuvier, 1832); skipjack tuna, *Katsuwonus pelamis* (Linnaeus, 1758); longtail tuna *Thunnus tonggol*, (Bleeker, 1851) and Pacific bluefin tuna, *T. orientalis* (Temminck & Schlegel, 1844). The post-thaw sperm of all five of these species had a duration of motility of 650 s and a motility rate of >70%, indicating that this simple method can be used to obtain high-quality cryopreserved sperm of various Scombridae species in outdoor environments.

Introduction

The Scombridae family, including mackerels, bonitos, and tunas, constitutes one of the most important fishery resources in the world. Because of this family's important commercial value, the recent development of aquaculture technology has shown great interest in its fish species. This interest has resulted in the establishment of full cycles of cultivation of various Scombridae species, such as the chub mackerel, *Scomber japonicas* (Houttuyn, 1782), (Murata et al., 2005); Pacific bluefin tuna, *Thunnus orientalis* (Temminck & Schlegel, 1844), (Sawada, Okada, Miyashita, Murata & Kumai, 2005); and eastern little tuna, *Euthynnus affinis* (Cantor, 1849), (Yazawa et al., 2015, 2016, 2017).

This achievement of full life cycles opens the opportunity for genetic improvements in Scombridae species, aimed at improving aquaculture productivity and commercial value. Considering this, future demand for the development of new strains carrying desirable genetic traits is expected to increase. Hybridization is one of the most common breeding techniques used in the agricultural and livestock industries (Kingsbury, 2009) and combines desirable traits of two different parent species into an offspring. In addition, the hybrids produced by a certain pairing have more desirable traits than those of both their parents, through a process called positive heterosis (Lippman & Zamir, 2007).

However, in cases of inter-species hybridization, it can be difficult to obtain both gametes simultaneously, because of temporal and spatial mismatches in maturation between the two parent species. In these situations, if it is possible to cryopreserve either the eggs or the sperm until the time of fertilization; such issues may be resolved. For decades now, cryopreservation techniques for fish sperm have been established in various species (Suquet, Dreanno, Fauvel, Cosson & Billard, 2000; Cabrita et al., 2010). However, effective cryopreservation has not been established for fish eggs, because fish eggs are relatively large

contains much lipid and egg yolk (Mazur, Leibo & Seidel, 2008). Consequently, the typical approach to creating a hybrid has been to cryopreserve sperm and use it to fertilize fresh eggs obtained from the female parent species during their maturational season.

The most common method for cryopreserving fish sperm is the floating-frame method, wherein a straw filled with semen is frozen on a frame floating on the surface of liquid nitrogen (Suquet et al., 2000). This method optimizes the cooling rate according to each individual fish species, by changing the distance between the surface of the liquid nitrogen and the semen-filled straw (i.e., the height of the floating-frame).

However, producing high-quality cryopreserved sperm from wild Scombridae fish requires performing the freezing operation in unstable outdoor environments, for instance at fishing ports or onboard fishing boats. In such cases, the floating-frame method cannot realize the optimum cooling rate and stable cryopreservation, because of the influence of ambient temperatures, strong winds, and movement aboard boats. In addition, this method requires liquid nitrogen, and the environments described above may not have access to it, or it may be impossible to transport it there.

For the above reasons, our study focuses on a sperm cryopreservation method suitable for freezing operations in outdoor environments: the dry-shipper method. A dry-shipper is a container used to transport biological materials at cryogenic temperatures (i.e., <−150°C). Dry-shippers have absorbent material on the inside of the container, which absorbs liquid nitrogen and enables it to keep the contents dry at cryogenic temperatures. A sperm cryopreservation method using dry-shipper was developed by the World Fisheries Trust in 1990, and it has been used to cryopreserve the sperm of salmonid species native to Canada, with the aim of genetic resource preservation (Harvey, 2000). Recently, it has also been reported that this method has been used for sperm cryopreservation in endangered freshwater fish species, such as the Characiformes and Siluriformes (Carolsfeld, Godinho, Zaniboni

Filho & Harvey, 2003; Viveiros & Godinho, 2009). However, only a limited number of studies have reported its use in marine fish species (Koh et al., 2013), and no study has reported the use of this method on wild, marine fish species in unstable outdoor environments.

To bridge this gap, our study proposes a sperm cryopreservation method for Scombridae species using a dry-shipper, which is suitable for freezing operations in the outdoor environments. First, a preliminary study determined optimum conditions for sperm cryopreservation using sperm from bullet tuna, *Auxis rochei* (Risso, 1810). Second, we selected five Scombridae species: chub mackerel; blue mackerel, *Scomber australasicus* (Cuvier, 1832); skipjack tuna, *Katsuwonus pelamis* (Linnaeus, 1758); longtail tuna, *Thunnus tonggol* (Bleeker, 1851); and Pacific bluefin tuna for practical trials of the dry-shipper method. Specimens of all five species were caught in various outdoor environments, and the sperm obtained was cryopreserved under optimized conditions. Based on our findings, we confirm that the dry-shipper method can be applied to Scombridae fish species, allowing a simple and stable production of high-quality cryopreserved sperm, even in outdoor environments.

Materials and methods

Ethics

All experiments were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals of the Tokyo University of Marine Science and Technology, Tokyo, Japan.

Sample fish for optimizing cryopreservation conditions

Bullet tuna were caught in Tateyama-shi, Chiba, Japan, from July 11–13, 2015. Twelve mature males were used as sample fish in the following experiments [fork length (FL): 35.2 ± 1.1 cm; body weight (BW): 734.4 ± 63.3 g; gonad weight (GW): 40.3 ± 4.5 g]. Immediately after the sample fish had been caught, semen was collected from each male by gently squeezing the abdomen. The collected semen was immediately cryopreserved at the sampling site (i.e., fishing ports), where the air temperature was approximately 26°C. The concentration of sperm in each semen sample (diluted if necessary) was counted using a hemocytometer.

Sperm cryopreservation

A dry-shipper (VOYAGEUR 5; Air Liquide, Paris, France) that had been completely filled with liquid nitrogen one day before sampling was used for sperm cryopreservation and transport. Semen collected from the sampled fish was diluted with an ice-cold cryomedium (consisting of an extender and a cryoprotectant, as described in chapter "Determining optimum extender and cryoprotectant conditions") at a ratio of 1:20 (v:v). The diluted semen was equilibrated on ice for 10 min and dispensed into a 0.5 mL straw (Fujihara Kogyo, Tokyo, Japan), which was then sealed with straw powder (Fujihara Kogyo, Tokyo, Japan) (Fig. 1a). Up to eight straws containing diluted semen were placed in a goblet (PA003 13 mmφ; My Science, Tokyo, Japan), and up to two goblets were attached to a single cryocane (C-2; My

Science, Tokyo, Japan) and frozen in the dry-shipper (Fig. 1b and c). All of the cryopreserved sperm was transferred into a liquid nitrogen storage tank and stored for 24 h. Following this, samples were thawed, and sperm motility was assessed as described in chapter "Thawing of sperm and evaluation of sperm motility".

Determining optimum extender and cryoprotectant conditions

The performance of four types of extenders used to cryopreserve the sperm of tuna: 300 mM trehalose (Miyaki, Nakano, Ohta & Kurokura, 2005), fetal bovine serum (FBS; A31606; Thermo Fisher Scientific, MA, USA) (Koh, Yokoi, Tsuji, Tsuchihashi & Ohta, 2010; Ohta, 2011), Ringer's solution (consisting of 141.0 mM NaCl, 5.2 mM KCl, 4.9 mM $CaCl_2$, 1.1 mM $MgCl_2$, 1.8 mM NaH_2PO_4, and 10 mM $NaHCO_3$ at pH 7.5) (Pillai, Yanagimachi & Cherr, 1994; Suquet et al. 2000), and 1% NaCl (Gwo, Weng, Fan & Lee, 2005). In this experiment, dimethyl sulfoxide (DMSO) was used as an internal cryoprotectant, so the cryomedium was composed of 90% (v/v) extender and 10% (v/v) DMSO.

To determine the optimum cryoprotectant, the performance of two compounds was assessed: DMSO (Suquet et al., 2000), which has been used to cryopreserve the sperm of a wide variety of fish species, and methanol (MeOH) (Harvey, Kelley & Ashwood-Smith, 1982; Tsutaka, Takii, Yamamoto & Ohta, 2006), which has proven effectiveness for sperm cryopreservation in some freshwater fish species. Three concentrations of DMSO [5%, 10%, and 20% (v/v)] and one concentration of MeOH [10% (v/v)] were added to the cryomedium with FBS. The concentration of MeOH was determined according to a report by Tsutaka et al. (2006).

Effect of freezing position on cryopreserved sperm quality

This experiment was aimed at investigating the effects of the freezing position in the dry-shipper on the motility of cryopreserved sperm once the sperm had been thawed. The semen from a single individual was frozen with two goblets attached to the upper and lower parts of the cryocane, and the motility of these cryopreserved sperms between the upper and lower parts was compared. These samples were mixed with a cryomedium consisting of 90% FBS and 10% DMSO, and each goblet contained eight straws filled with diluted semen. Five of the eight straws in each goblet were then randomly selected and thawed to evaluate sperm motility as described in chapter "Thawing of sperm and evaluation of sperm motility".

Comparison with the floating-frame method

This experiment compared the motilities of post-thaw sperm obtained from a single male and cryopreserved using the floating-frame and sperm obtained using dry-shipper methods. For the floating-frame method, cryopreserved sperm was obtained according to the method in Gwo et al. (2005) for Pacific bluefin tuna. Straws with diluted semen were prepared and frozen in liquid nitrogen vapor in a foamed polystyrene box containing liquid nitrogen to a depth of 3–4 cm. The straws were placed on a 2 cm high foamed polystyrene frame floating on the surface of the liquid nitrogen. Freezing time was 10 min, after which the straws were plunged directly into the liquid nitrogen. Simultaneously, the other half of the prepared straws were frozen using the dry-shipper method, following the aforementioned procedure. Both experiments used 90% FBS and 10% DMSO as a cryomedium, and cryopreserved sperm was stored in a liquid nitrogen storage tank.

Thawing of sperm and evaluation of sperm motility

Straws were removed from the liquid nitrogen storage tank and immersed in a 1 L beaker filled with tap water at approximately 19°C for 10 s. Post-thaw sperm was then activated by

adding 950 μL of sterile seawater at 24°C to each 50 μL of diluted semen samples. To allow clear observations, the sterile seawater solution was complemented with 0.5% of bovine serum albumin, to prevent the physical adsorption of sperm onto the cover glass. The duration of sperm motility and the sperm motility rate were measured under an optical microscope (BX51N-34FL; Olympus, Tokyo, Japan). The duration of sperm motility was taken as the time period from the activation of the sperm to the point when none of the sperm in the microscopic field exhibited forward motility. The sperm motility rate was measured according to the method reported by Tsutaka et al. (2006), whereby frame-by-frame images were recorded from 60 to 65 s after sperm activation using a digital camera (DP70; Olympus, Tokyo, Japan) installed on the optical microscope. More than 50 sperms were then randomly selected in the recorded frame-by-frame images and examined to determine whether they moved forward or had no movement. The sperm motility rate was then calculated as the ratio of forward-moving sperm cells to the total number of selected sperm cells. We observed the cryopreserved sperm of three individuals under each test condition and calculated their average motilities for comparison.

Fertilization test

Approximately 15,000 unfertilized eggs were obtained by gently squeezing the abdomen of a female bullet tuna (FL: 35.8 cm; BW: 731.5 g; GW: 28.2 g) caught in Tateyama-shi, Chiba, on July 27, 2015. Unfertilized eggs were equally divided among four 1 L beakers and artificially inseminated with fresh sperm from a bullet tuna male (FL: 34.2 cm; BW: 662.1 g; GW: 35.8 g) caught on the same day and cryopreserved sperm from three males (FL: 34.5 ± 1.4 cm; BW: 721.6 ± 61.2 g; GW: 39.1 ± 4.4 g) frozen using the dry-shipper method (cryomedium: 90% FBS, 10% DMSO) on July 13, 2015. The artificial insemination procedure was as follows: 150 μL of fresh sperm (approximately 4.2×10^9 sperm cells) or

500 μL of cryopreserved sperm (approximately 7.0×10^8 sperm cells) were added to approximately 3,750 unfertilized eggs and mixed together. Then, 1 L of sterile seawater at 24°C was added in each beaker, to induce fertilization. Eggs that rose to the water surface within 5 min of fertilization were defined as floating eggs and the floating egg rate was calculated as [floating egg count / total eggs used for each experimental group × 100]. Triplicate samples of 30 floating eggs were collected into 8-mL Petri dishes that were then filled with sterile seawater and incubated at 24°C. The fertilized egg count was then determined as the number of eggs that reached the two- to four-cell stages within 1–2 h of fertilization, and the hatched egg count was calculated as the number of larvae that hatched within 48 h of fertilization. The fertilization rate was then calculated as [(fertilized egg count / 30 floating eggs × 100) × floating egg rate (%)], and the hatching rate was calculated as [(hatched larva count / 30 floating eggs × 100) × floating egg rate (%)].

Production of cryopreserved sperm: blue mackerel and chub mackerel

Cryopreserved sperms from blue mackerel and chub mackerel were produced on June 20 and June 22, 2015, respectively. Three male chub mackerels (FL: 34.9 ± 1.1 cm; BW: 560.5 ± 33.6 g; GW: 30.0 ± 6.0 g) and three male blue mackerels (FL: 32.8 ± 0.7 cm; BW: 545.3 ± 63.8 g; GW: 32.5 ± 2.4 g) reared in a 10 t land-based tank at the Tateyama Station (Banda), Field Science Center of Tokyo University of Marine Science and Technology (Chiba, Japan) were used as fish samples. On the day before the production of cryopreserved sperm, 100 U/kg of human chorionic gonadotropin (hCG; gonatropin 5000; Aska Pharmaceutical, Tokyo, Japan) was injected into each individual to induce sperm production. Semen was then collected from each individual by gently squeezing the abdomen, 38–42 h after the injection. Collected semen samples were cryopreserved with the cryomedium (90% FBS and 10% DMSO). The freezing operation was performed outdoors, where the air

temperature was 22°C. Immediately after production, the cryopreserved sperm was transferred into a liquid nitrogen storage tank and stored for 24 h. Following this, samples were thawed, and sperm motility was evaluated, as described in chapter “Thawing of sperm and evaluation of sperm motility”.

Production of cryopreserved sperm: skipjack tuna

Three male skipjack tunas weighing 7–8 kg caught in Amami-shi, Kagoshima, Japan, on June 27, 2015, were used as samples. As we were unable to obtain semen by squeezing the individuals’ abdomen, the testes from each individual we dissected to allow semen to be collected directly from the vas deferens. The freezing operation was performed at the dissection site (i.e., the fishing port) at an air temperature of 28°C using the cryomedium described earlier (90% FBS and 10% DMSO). The cryopreserved sperm was transported to the Tateyama Station using a dry-shipper (transportation time = 1 day) and then transferred into a liquid nitrogen storage tank and stored for 24 h. Following this, samples were thawed, and sperm motility was evaluated, as described in chapter “Thawing of sperm and evaluation of sperm motility”.

Production of cryopreserved sperm: longtail tuna

Three male longtail tunas (FL: 59.0 ± 6.2 cm; BW: 3.9 ± 0.5 kg; GW: 27.3 ± 5.3 g) caught by hook and line in Nagato-shi, Yamaguchi, Japan, on August 30, 2015, were used as samples. As with the skipjack tuna, semen was collected directly from the vas deferens of the individuals. Freezing was performed onboard the boat at an air temperature of 24°C using a cryomedium (90% trehalose (300 mM) and 10% DMSO). This differed from the cryomedium used for the other species because FBS cannot be stored for a long time at room

temperature, whereas trehalose powder can. Therefore, 300 mM trehalose was prepared by mixing trehalose powder with distilled water at the sampling site and was used as an extender for the longtail tuna sperm samples. The cryopreserved sperm was then transported to the Tateyama Station using a dry-shipper (transportation time = 1 day), where it was transferred into a liquid nitrogen storage tank and stored for 24 h. Thereafter, samples were thawed, and sperm motility was evaluated, as described in chapter “Thawing of sperm and evaluation of sperm motility”.

Production of cryopreserved sperm: Pacific bluefin tuna

Three 3- to 4-year-old male Pacific bluefin tunas cultivated in net pens in Kushimoto-cho, Wakayama, Japan, were captured on August 12, 2015, to be used as samples. Similarly to that of longtail and skipjack tuna, the semen could not be obtained by squeezing the abdomen, so semen was collected directly from the vas deferens of each individual. Freezing was performed at the fishing port, at an air temperature of 30°C using a cryomedium containing 90% trehalose (300 mM) and 10% DMSO. Trehalose was used as an extender because it can be stored in powder form at room temperature, as explained in section 2.4.3. Cryopreserved sperm samples were transported to the Tateyama Station using a dry-shipper (transportation time = 1 day) and then transferred into a liquid nitrogen storage tank and stored for 24 h. Following this, samples were thawed, and sperm motility was evaluated, as described in chapter “Thawing of sperm and evaluation of sperm motility”.

Statistical analysis

All data are presented as mean values ± standard deviations. Statistical significance was determined using one-way ANOVA, followed by Tukey’s multiple comparison test, using a statistical significance level of $p < 0.05$. All analyses were carried out using GraphPad Prism

version 5.0 (GraphPad).

Results

Optimum conditions for cryopreserving bullet tuna sperm using the dry-shipper method

All samples had approximately 500 s of time duration of sperm motility and approximately 80% motility rate, with no significant difference in either measure between the four extenders (Fig. 2). Sperm frozen by the 10% DMSO cryoprotectant had 614 ± 18 s of time duration of sperm motility and 83.7 ± 0.5% motility rate (n = 3), which was significantly higher than that of sperm frozen using other conditions of cryoprotectants (Fig. 3). There was no significant difference in the motility rate of post-thaw bullet tuna sperm between the upper and lower parts of the freezing positions on the cryocane (duration of sperm motility = 580 ± 19 s and 588 ± 20 s, respectively; motility rate = 89.2 ± 3.0% and 88.9 ± 3.9%, respectively; n = 5 for each freezing position). Furthermore, the standard deviations of the duration of sperm motility and the motility rate for sperm that had been selected randomly from each freezing position were also small (Fig. 4).

Comparison between the dry-shipper method and the floating-frame method

The time duration of sperm motility and motility rate of the sperm produced by the dry-shipper method were 782 ± 92 s and 93.0 ± 7.3% (n = 3), respectively. By contrast, the time duration of sperm motility and the motility rate of sperm produced by the floating-frame method were 747 ± 59 s and 91.0 ± 5.5% (n = 3), respectively. The results were almost the same in both methods, with no significant difference detected (Fig. 5). The fertilization and hatching rates of eggs that were artificially inseminated with fresh sperm or cryopreserved sperm derived from three individuals are summarized in Table 1.

Cryopreservation of sperm from five species in the family Scombridae using the dry-shipper method

In addition to bullet tuna, sperm samples from five Scombridae species (blue mackerel, chub mackerel, skipjack tuna, longtail tuna, and Pacific bluefin tuna) were cryopreserved using the optimized dry-shipper method under various outdoor environments. The durations of sperm motility and motility rates of sperm from each species after cryopreservation and thawing are presented in Table 2. The duration of sperm motility ranged from approximately 600–1,200 s for all species, except for longtail tuna (approximately 3,600 s), and the motility rate was ≥ 70% across all the species.

Discussion

In this study, we cryopreserved sperm from six Scombridae species using the dry-shipper method in a range of outdoor environments. In all species, cryopreserved sperm had a high motility rate of ≥70% and 650 s duration of motility after samples had been thawed. These results indicate that the dry-shipper method for cryopreservation can be applied to various Scombridae species and can easily produce high-quality cryopreserved sperm even in unstable outdoor environments, in which the more traditional floating-frame method cannot be used.

The results indicated that 10% DMSO was the most effective internal cryoprotectant, but all four extenders resulted in similarly high motility rates. As duration of motility obtained with FBS and trehalose exhibited slightly more desirable results, we used a 90% FBS and 10% DMSO solution or a 90% trehalose (300 mM) and 10% DMSO solution as a cryomedium to produce cryopreserved sperm in the dry-shipper method.

It has previously been reported that FBS can be used to cryopreserve the sperm of a wide variety of freshwater and marine fish species (Ohta, 2011) but unfortunately it cannot be stored for a long time at room temperature, whereas 300 mM trehalose can be prepared by mixing trehalose powder (which can be stored at room temperature) with distilled water at the sampling site. Therefore, we used cryomedium containing 90% FBS and 10% DMSO for the production of cryopreserved sperm for most of the species tested and 90% trehalose (300 mM) and 10% DMSO when sampling was carried out under conditions where ice-cold transportation was practically impossible (i.e. for longtail tuna and Pacific bluefin tuna in the present study).

Sperm cryopreservation using the dry-shipper method is a simple procedure whereby straws are filled with diluted semen samples and inserted into a dry-shipper. We found that the freezing position in the dry-shipper did not affect the motility of cryopreserved sperm.

Thus, this method requires no expert skills and can be used for the simple and stable production of high-quality cryopreserved sperm.

Cryopreserved sperm from bullet tuna frozen using the dry-shipper method exhibits similar high motility to that of sperm frozen using the floating-frame method. In addition, we found that bullet tuna sperm that had been frozen using the dry-shipper method were able to fertilize freshly collected bullet tuna eggs, which subsequently hatched normally. Moreover, in all five species examined, the post-thaw sperm had a duration of motility of 650 s and a motility rate of ≥70%, which fell within the range observed for the sperm of Brazilian freshwater fish species that had been cryopreserved using the same method (20%–100%; Viveiros & Godinho, 2009). Thus, the dry-shipper method that was used in this study is suitable for the production of high-quality cryopreserved sperm of various Scombridae species that can be used for artificial insemination procedures, even in unstable outdoor environments where the more traditional floating-frame method cannot be used.

The dry-shipper container used in this study enabled the cryogenic environment to be maintained for 14 days, as it had been completely filled with liquid nitrogen. In reality, the production of cryopreserved sperm was the longest for skipjack tuna, which required a 4-day sampling period. In this case, simply filling the dry-shipper with liquid nitrogen once prior to departure allowed sperm cryopreservation and transportation to be achieved during the sampling period. The dry-shipper is also permitted by the International Air Transport Association to be loaded into airplanes, enabling sampling to be carried out at sites far from laboratories. Accordingly, the use of this method allows sampling to be completed even in isolated lands or in remote areas, regardless of whether they are in domestic or international waters, over a period of approximately two weeks. Thus, as rare and endangered species often inhabit places where liquid nitrogen is challenging to obtain, this method is useful for gene resource preservation technology.

This is the first study to produce cryopreserved sperm from bullet tuna, skipjack tuna, and longtail tuna. The duration of sperm motility in five of the six species examined ranged from approximately 600–1,200 s. By contrast, the duration of sperm motility in longtail tuna was approximately 3,600 s, which was approximately four times longer than that of Pacific bluefin tuna in the same genus. Thus, this method, which allows for the relatively easy collection of sperm from a wide variety of fish species, is useful for comparing the morphology and motility of sperm between several fish species and for studying their evolution. It should be noted, however, that these interspecific differences require further verification, as both the duration of sperm motility and motility rate vary at the peak of the spawning period (Mylonas, Papadaki & Divanach, 2003).

We previously established a method for inducing spawning in eastern little tuna in a land-based tank by administering gonadotropin-releasing hormone agonists (GnRHa) (Yazawa et al., 2015). Thus, artificial insemination with unfertilized eggs from eastern little tuna acquired using this technique and cryopreserved sperm from various other species in the Scombridae family would allow the production of hybrids for the first time. A method for artificial seed production of Pacific bluefin tuna by artificial insemination using eggs and sperm collected from fish before shipping has also been reported (Endo et al., 2016); thus, the combination of this method with cryopreserved sperm produced in this study is expected to allow the hybridization of Pacific bluefin tuna. Thus, it is expected that using the dry-shipper method will make it possible to create new hybrids that were once considered impossible with the previous techniques.

In this study, we demonstrated that the dry-shipper method allows for the simple production of high-quality cryopreserved sperm from various fish species in the Scombridae family, in any working environment. Therefore, it is expected that this method can also be applied to various other marine fish species and may possibly evolve into a breeding support

technique for cross-breeding and lineage conservation, as well as a conservation technique for endangered fish species.

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Figure legends

Figure 1. Cryopreservation of sperm using a dry-shipper.

(a) Straws filled with 0.5 mL of diluted semen, (b) a goblet held by a cryocane, and (c) storage of straws filled with sperm in a fully charged dry-shipper (VOYAGEUR 5, Air Liquide).

Figure 2. Effects of various extenders contained in the cryomedium on the motility of bullet tuna sperm.

(a) Duration of motility and (b) motility rate of post-thaw sperm with the medium containing 10% DMSO and 90% various extenders (300 mM trehalose, Ringer's solution, 100% FBS, or 1% NaCl; $n = 3$). Data are presented as mean ± SD.

Figure 3. Effects of various cryoprotectants contained in the cryomedium on the motility of bullet tuna sperm.

(a) Duration of motility and (b) motility rate of post-thaw sperm with the cryomedium containing FBS and MeOH or DMSO at various concentrations (10% MeOH; 5%, 10%, or 20% DMSO; $n = 3$, * $p < 0.05$, Tukey–Kremer test). Data are presented as mean ± SD.

Figure 4. Effects of two freezing positions on the motility of bullet tuna sperm.

(a) Duration of motility and (b) motility rate of post-thaw sperm in an upper goblet and a lower goblet ($n = 5$) with the cryomedium containing 90% FBS and 10% DMSO. Data are presented as mean ± SD.

Figure 5. Effects of two freezing methods on the motility of bullet tuna sperm.

(a) Duration of motility and (b) motility rate of post-thaw sperm with the conventional

floating-frame method or dry-shipper method (n = 3) with the cryomedium containing 90% FBS and 10% DMSO. Data are presented as mean ± SD.

Fig. 1

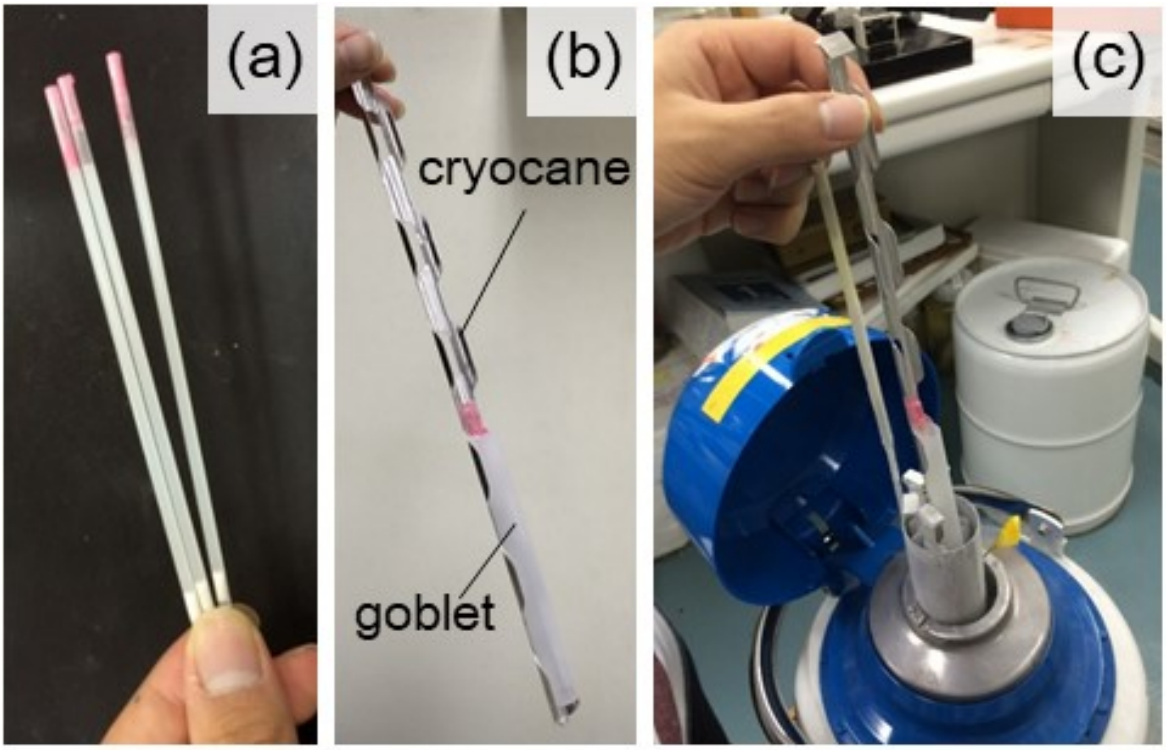


Fig. 2

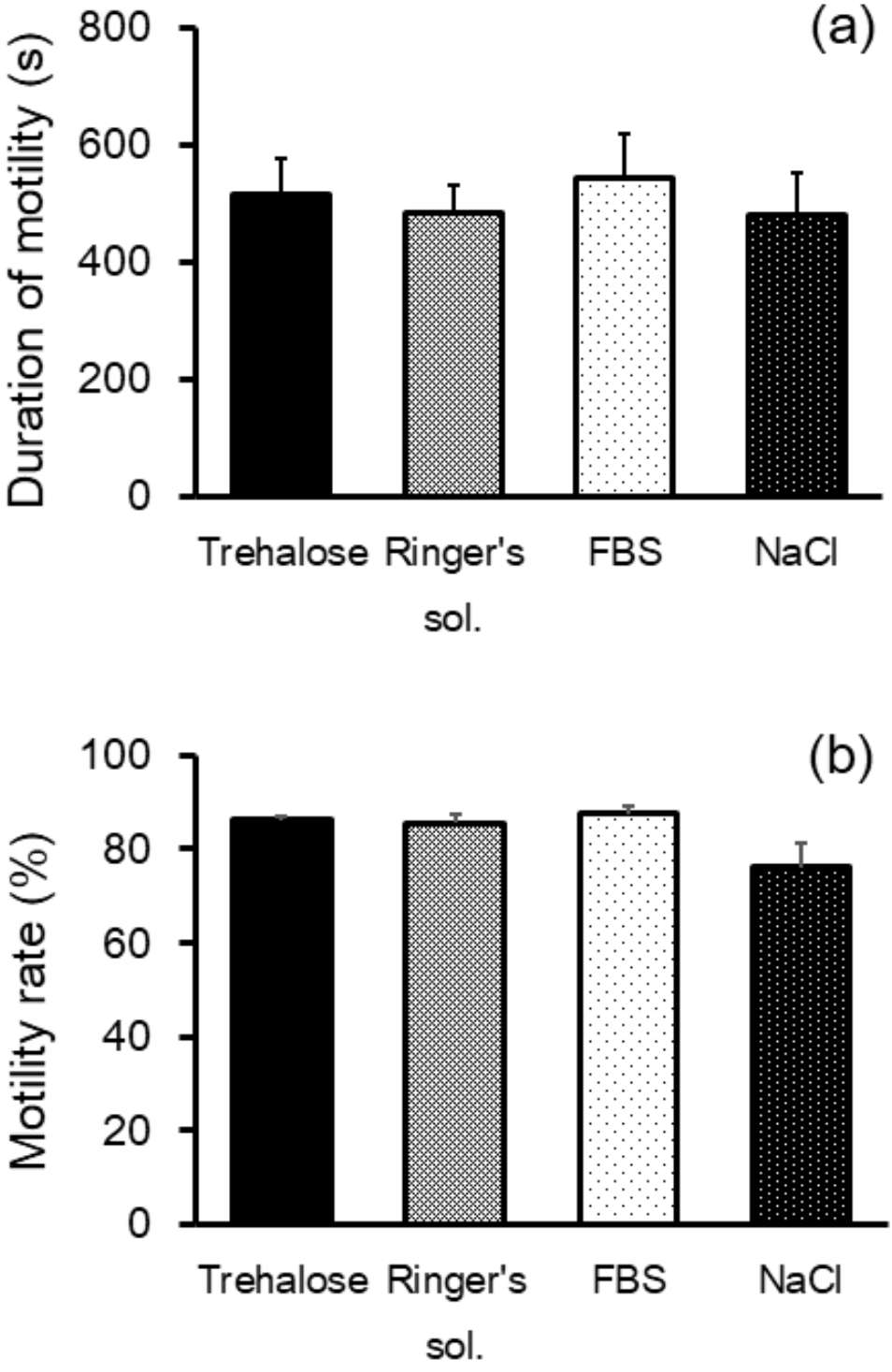
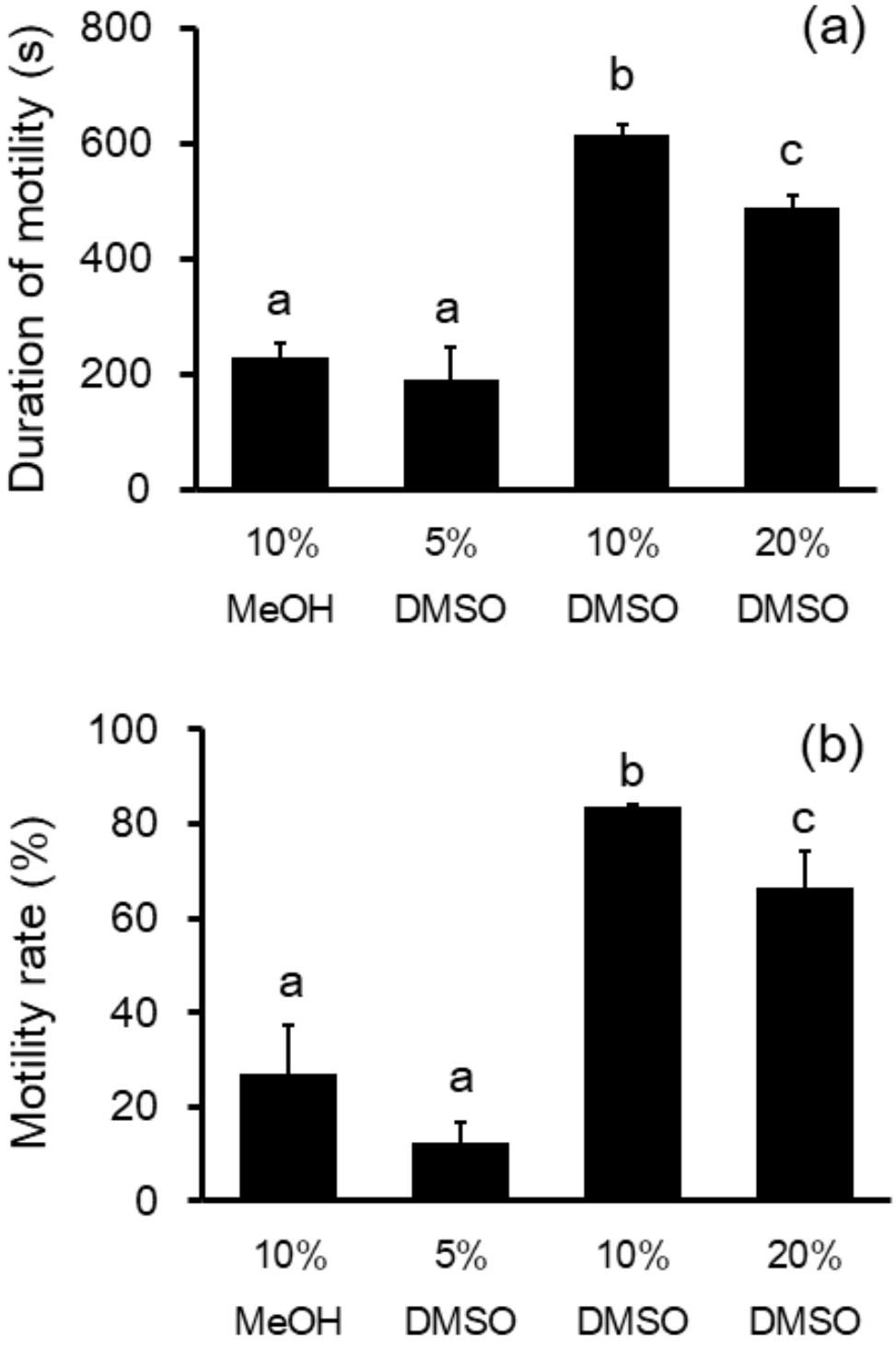

(a)
Duration of motility (s)
800
600
400
200
0
Trehalose
Ringer's sol.
FBS
NaCl
(b)
Motility rate (%)
100
80
60
40
20
0
Trehalose
Ringer's sol.
FBS
NaCl

Fig. 3


(a)
Duration of motility (s)
800
600
400
200
0
a
a
b
c
10% MeOH
5% DMSO
10% DMSO
20% DMSO
(b)
Motility rate (%)
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c
10% MeOH
5% DMSO
10% DMSO
20% DMSO

Fig. 4

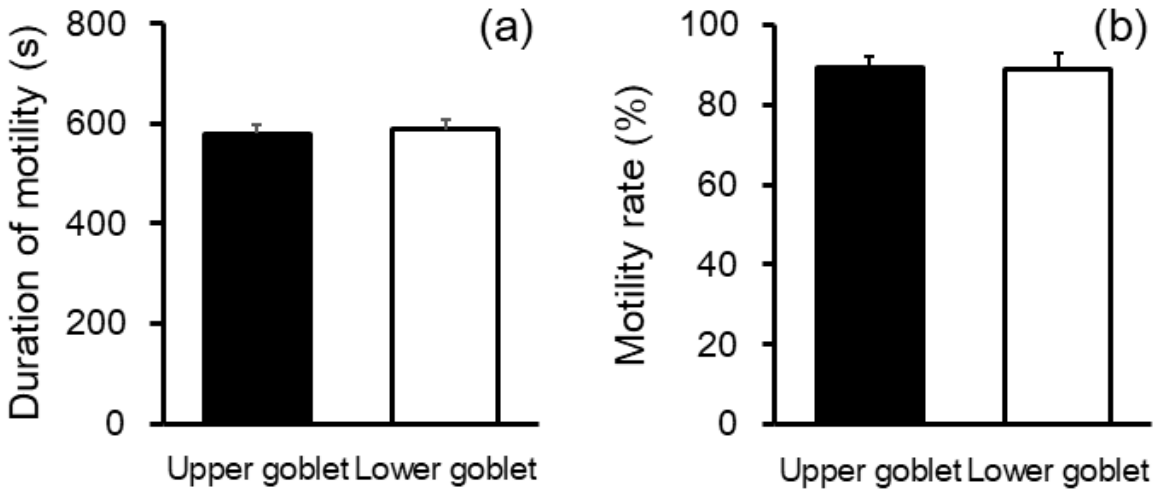
(a)
Duration of motility (s)
800
600
400
200
0
Upper goblet
Lower goblet
(b)
Motility rate (%)
100
80
60
40
20
0
Upper goblet
Lower goblet

Fig. 5

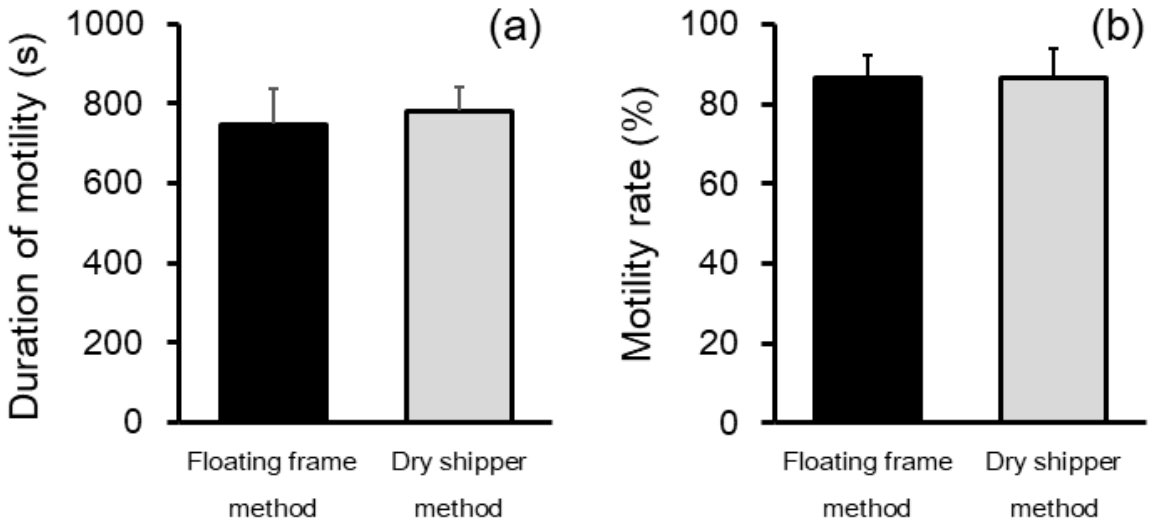
(a)
Duration of motility (s)
1000
800
600
400
200
0
Floating frame method
Dry shipper method
(b)
Motility rate (%)
100
80
60
40
20
0
Floating frame method
Dry shipper method

Table 1. Fertilization and hatching rates of eggs inseminated with cryopreserved and fresh bullet tuna sperm.

	Cryo. #1	Cryo. #2	Cryo. #3	Fresh sperm
Fertilization rate	25.3%	27.7%	24.1%	21.7%
Hatching rate	14.1%	18.5%	13.9%	12.7%

Table 2. Duration of motility and motility rate of post-thaw sperm of six Scombridae species frozen by dry-shipper method.

Species	No. of samples	Extender	Sperm motility	
			Duration of motility (s)	Motility rate (%)
Blue mackerel	3	100% FBS	1,028 ± 27	88.9 ± 2.5
Chub mackerel	3	100% FBS	722 ± 50	71.4 ± 5.7
Skipjack tuna	3	100% FBS	845 ± 219	79.4 ± 6.1
Bullet tuna	3	100% FBS	782 ± 59	86.6 ± 9.0
Pacific bluefin tuna	3	300 mM Trehalose	789 ± 46	78.4 ± 3.2
Longtail tuna	3	300 mM Trehalose	3,510 ± 295	70.8 ± 4.8

All specimens were frozen with cryomedium containing 90% extender (100% FBS or 300 mM trehalose) and 10% DMSO. Data are presented as mean ± SD.

第 3 章

スマ雑種の作出とその特性解析

緒言

代理親魚技法によって宿主にドナー由来の配偶子を高効率で生産させるためには、ドナー種に合わせて適切な宿主種を選択することが重要である。一般に、宿主種とドナー種が遺伝的に近縁であるほど宿主のドナー由来配偶子の生産効率が高い（Bar et al., 2016; Yoshizaki and Yazawa, 2019; Goto and Saito, 2019）。また、産卵適水温等の繁殖に関わる生理学的特性が宿主種とドナー種で類似しているかどうかも、宿主によるドナー由来配偶子の生産効率に関わる重要な要素であると考えられる。以上の観点から、クロマグロの宿主候補としてスマ *Euthynnus affinis* が最有力であると期待されている。スマの属するサバ科スマ属は、クロマグロが属するサバ科マグロ属と遺伝的に最も近縁な関係にある分類群であり（Miya et al., 2013）、インド太平洋の熱帯から亜熱帯に生息するスマは産卵適水温等の生理学的特性がクロマグロと類似している（Yesaki, 1994）。さらに、実際にマサバ、ハガツオ、スマの仔魚へクロマグロ精原細胞を移植したところ、3 種の中でスマは最も高いクロマグロ精原細胞の取り込み効率を示した（Yazawa et al., unpublished data）。したがって、第 1 章で作出したゴマサバ×マサバ雑種のようなスマの不妊雑種を作出することができれば、クロマグロ配偶子を高効率で生産する宿主になることが期待される。

また、矢澤らによる一連の研究によって、スマは飼育下で満 1 歳、体重 1.5 kg で成熟可能であることが明らかにされているうえ、半循環式陸上 10m^3 水槽を用いた周年採卵技術が構築されている（Yazawa et al., 2015; 2016; 2017）。したがって、スマを宿主に用いたクロマグロ代理親魚技法が構築できれば、前述したサバと同様に親魚飼育にかかるコストを大幅に削減できるだけでなく、短期間で成熟することによるクロマグロ育種の高速化や周年採卵技術を利用したクロマグロの早期種苗生産が可能となり、養殖クロマグロの戦略的な育種において有用な技術になることが期待される。

以上の背景から第 3 章ではスマ雑種の作出を試みた。しかし、種間交雑では交雑に用いる親魚種の組み合わせによって雑種致死や雑種弱勢が生じることが報告されている（Arai, 1984; 伊藤ら 2006）。したがって、生残性の雑種を得るためには、様々な交雑の組み合わせを検討する必要がある。そこで、スマのメス親魚から得た卵に対して、第 2 章で作製した 3 属 4 種（カツオ、マルソウダ、コシナガ、クロマグロ）の凍結精子を媒精し、生残性の雑種が得られるかどうかを調べた。

材料および方法

親魚養成

スマ親魚の養成は、北緯 34.97 東経 139.761 に位置する東京海洋大学館山ステーションで行った。飼育水槽はテントハウス（株式会社ニッケーコー）内に設置した FRP 製 70 m^3 陸上水槽（アース株式会社）およびコンクリート製 38 m^3 陸上水槽を用いた。飼育水には FRP 製自動逆洗砂ろ過機（FST-3000,ヤンマー造船株式会社）により濾過した海水を用い、70 m^3 水槽の注水量は 80 L/min で 1 日 1.6 回転とし、38 m^3 水槽の注水量は 70 L/min で 1 日 2.7 回転とした。70 m^3 水槽の照明には 70 W メタルハライドランプ（ファンネルラッキー70 W 20000 K, 神畑養魚株式会社）2 基を用い、38 m^3 水槽の照明には 70 W メタルハライドランプ 1 基および 32 W スパイラル蛍光灯（TSW-32W, トラスコ中山株式会社）2 基を用いた。照明の点灯時間は 24 時間明期とした。餌には適当なサイズに切り分けたツナチューブ（日本水産株式会社）、冷凍サバ、冷凍イカナゴおよび冷凍オキアミを用い、1 日の給餌量は魚体重あたり 5-10%とした。また、水槽搬入時およびホルモン投与時に胸鰭の一部をサンプリングし、Yazawa et al.（2015）の方法に従ってエストラジオール 17β（E2）および 11-ケトテストステロン（11-KT）を酵素免疫測定（ELISA）法によって測定し雌雄の判別を行った。

ホルモン投与

Amezawa et al. 2018 の方法に従い、以下の手順で生殖腺刺激ホルモン放出ホルモンアナログ（GnRHa）のコレステロールペレットを作成した。GnRHa は Anygen 社（Jeollanam-do, Korea）のカスタムオーダーサービスにより合成した。15mg の GnRHa を 3 ml の 70%エタノール（和光純薬株式会社）に溶解し、3,750 mg のコレステロール（和光純薬株式会社）を加えて混合した後、室温で 30 分間乾燥させた。

さらに 750 mg の溶解させたココアバターを加えて混合し、室温で一晩乾燥させた。調整した粉末をアクリル製のペレット作成器を用いて、1 粒あたり GnRHa を 150 μg 含有するコレステロールペレット（直径 3 mm, 長さ 5 mm, 重さ 45 mg）を作成した。Yazawa et al.（2015）の方法に従い、GnRHa が魚体重に対して 0.1 mg/kgBW となるように、スマ親魚の背側の筋肉内へ GnRHa コレステロールペレットを投与して排卵誘発を行った。オス親魚の排精促進には、ヒト絨毛性生殖腺刺激ホルモンである HCG（human chorionic gonadotropin, あすかアニマルヘルス株式会社）を使用した。オス親魚に対する HCG の投与量は 100 IU/kgBW とし、背筋部に注射投与した。

人工授精

GnRHa コレステロールペレットによって産卵誘発されたスマはおよそ 24 時間周期で数日間にわたって産卵が継続する。そこで、産卵誘発されたスマの排卵時刻を推定するために、ホルモン投与後 2 日間の産卵時刻を記録した。人工授精はホルモン投与後 3 日目に行い、前日 2 日間の産卵時刻の直前を人工授精の実施時刻とした。人工授精の際は、水槽の水位を腰丈まで落とし、ラバーネット（PK704-19CRK, 株式会社プロックス）を用いてスマを捕獲した。海水が混入しないように総排出孔付近をキムタオル（日本製紙クレシア株式会社）で拭き取ったのち、腹部を圧迫することで卵を搾取した。2 L のプラスチックビーカー（アズワン株式会社）に卵を回収し、乾導法によって凍結精子と媒精した。凍結精子ストローの解凍は、26℃の水道水中で 10 秒間攪拌することで行い、はさみでストロー両端を切断してプラスチックビーカー内の卵と混合した。凍結精子ストローの使用本数は卵 2 万粒に対して 1 本（500 μL）を目安とした。卵と精子をよく混合した後、25-26℃に調温した自然海水を 2,000 ml 加え 5 分間静止した。その後、受精卵を自然海水で 3 回洗浄した。

卵質評価および受精から孵化までの観察

受精卵を 2,000 ml の海水中へ懸濁した後、2 ml を 6 穴プレート（TPP）へ移し、卵数を計測した。3 回計測した卵数の平均値に 1,000 を乗じた数を全卵数とした。次に、全卵数測定用に用いた 2 ml 中の卵のうち、5 分以内に水面に浮上した卵の数を浮上卵数として測定した。また、浮上卵を 30 粒ずつ 6 穴プレートの 3 区画へ移し、計 90 粒を 26℃のインキュベーター内で飼育した。飼育には各区画 8 ml のオートクレーブ滅菌海水を用いた。水質の悪化を防ぐため、飼育中は死卵を適宜回収し、受精後 1-2 時間で 2-4 細胞期に到達している卵数を受精卵数、13-14 時間で胚体形成している卵数を胚体形成卵数、30-31 時間で孵化している仔魚数を孵化数として計測した。さらに、受精率 （=受精卵数/90（6 穴プレートに移した全卵数）×100）と胚体形成率 （=胚体形成卵数/90×100）、孵化率 （=孵化仔魚数/90×100）を求めた。受精から孵化までの観察には、上述の孵化率・胚体形成率・孵化率測定用区画とは別に卵観察用区画を設け、卵割を開始した受精卵のみを 30-50 粒移した。その後、上述の卵数測定と同時に受精・胚体形成・孵化を実体顕微鏡（SZX12, オリンパス株式会社）で観察し、顕微鏡に装着した CCD カメラ（DP70, オリンパス株式会社）で撮影した。

仔魚飼育および形態観察

人工授精によって作出した受精卵は、浮上卵のみ 100 L サンライト水槽（SLP-100, 田中三次郎商店）に収容した。飼育水温は 25-28℃で、通気量は約 100 mL/min であった。照明は 150 W メタルハライドランプ（ファンネル 2, 神畑養魚株式会社）を用い、点灯時間は 14 時間明期、10 時間暗期とした。初期餌料にはシオミズツボワムシを用い、水槽内のワムシ密度が 10-20 個体/ml になるように 3 日齢から給餌を開始した。なお、シオミズツボワムシは、30 g/100 L/日のハイパーグリーン（日清マリンテック）により栄養強化を行った。また、ワムシの給餌開始後から飼育水に

10 mL/100 L のナンノクロロプシス（マリーンフレッシュ，マリーンバイオ）を 1 日 2 回添加した。孵化直後に底掃除を行い、水槽底面に沈降した卵殻や死卵を取り除いた。1 日齢から 8 日齢まで毎日サンプリングを行い、実体顕微鏡（SZX12, オリンパス株式会社）を用いて仔魚の形態観察および全長の測定を行った。なお全長は各日齢で 3 個体ずつ測定し、その平均値で各試験区の成長を比較した。

スマ×クロマグロ雑種の作出

株式会社丸東（和歌山県東牟婁郡串本町）の保有する海面生け簀で飼育され、活魚トラックによる陸上輸送で 2012 年 9 月 3 日に 70 m^3 陸上水槽へ搬入された後、1 年間養成した 3 歳魚のスマ 9 尾を親魚として用いた。クロマグロの凍結精子を用いた人工授精は 3 回実施した。実施日はそれぞれ 2013 年 10 月 24 日、10 月 31 日、11 月 15 日であった。

スマ×カツオ雑種、スマ×マルソウダ雑種、スマ×コシナガ雑種の作出

38 m^3 水槽にて館山ステーションで受精卵から養成した 1 歳魚のスマ 6 尾を親魚として用いた。カツオ、マルソウダ、コシナガの凍結精子には第 2 章で作製したものを用いた。ホルモン投与による産卵誘発は 2015 年 9 月 30 日および 2015 年 10 月 5 日に実施し、投与後 2 日間にわたって産卵時刻を確認した後、10 月 4 日の午前 4 時に 1 回目、10 月 8 日の午後 8 時 30 分に 2 回目の人工授精をそれぞれ実施した。産卵誘発を行ったスマ親魚群の中から、腹部の圧迫によって排卵が確認できたメス 1 尾を人工授精に用いた。また同時に、オス 1 尾から精子を採取し、交雑試験のコントロールとしてスマ×スマも作出した。10 月 4 日の人工授精では、スマのメス（全長 44.5 cm 尾叉長 42.5 cm 体重 1,553.3 g 生殖腺重量 31.3 g）から 126,333 粒の卵を搾取した。得られた卵を 3 試験区に分け、65,333 粒の卵に対してカツオの凍結精子ストロー3 本（精子数：4.2×10^8 細胞/本）、47,667 粒の卵に対してマルソウダ

の凍結精子ストロー3 本（精子数 : 3.7×10^8 細胞/本）および 13,333 粒の卵に対してスマのオス（全長 43.0 cm 尾叉長 41.1 cm 体重 1711.1 g 生殖腺重量 16.3 g）から搾取した 2 ml の精子をそれぞれ媒精させた。10 月 8 日の人工授精では、メス（全長 47.5 cm 尾叉長 45.5 cm 体重 1,721.2 g 生殖腺重量 23.1 g）から 169,334 粒の卵を搾取した。得られた卵を 4 試験区に分け、35,000 粒の卵に対してカツオの凍結精子ストロー2 本（精子数：4.2×10^8 細胞/本）、35,667 粒の卵に対してマルソウダの凍結精子ストロー2 本（精子数：3.7×10^8 細胞/本）、60,667 粒の卵に対してコシナガの凍結精子ストロー3 本（精子数：2.1×10^8 細胞/本）、38,000 粒の卵に対してスマのオス（全長 42.1 cm 尾叉長 40.5 cm 体重 1,678.4 g 生殖腺重量 15.1 g）から得た 2 ml の精子をそれぞれ媒精させた。

統計処理

スマおよび各雑種仔魚の全長は、平均値±標準偏差で示している。各日齢におけるスマとスマ×コシナガ雑種間およびスマとスマ×クロマグロ雑種間の有意差検定には GraphPad Prism Version 5.0（GraphPad, La Jolla, CA）を用いて、Welch の t 検定を行った。（$p < 0.05$）

結果

メスのスマから得た卵に 3 属 4 種（カツオ属カツオ、ソウダガツオ属マルソウダ、マグロ属コシナガ、マグロ属クロマグロ）の凍結精子を媒精させた交雑試験の結果を表 1 および図 1 のグラフに示す。媒精 1-2 時間後にコントロールのスマおよび全ての雑種で 2-4 細胞期の受精卵が観察された（図 2A, D, F, H, K）。各交雑試験の受精率（mean ± SEM）はスマが 77.0 ± 5.0%（n＝5）、スマ×カツオ雑種が 17.4 ± 2.7%（n＝2）、スマ×マルソウダ雑種が 50.8 ± 2.7%（n＝2）、スマ×コシナガ雑種が 53.0%（n = 1）、スマ×クロマグロ雑種が 55.0 ± 21.2%（n = 3）であった（図 1）。受精卵の発生を経時的に観察したところ、媒精 8-9 時間後にスマでは原腸陥入に伴う覆いかぶせ運動（エピボリー）が観察されたが（図 3A）、スマ×カツオ雑種およびスマ×マルソウダ雑種では胞胚期からエピボリー期にかけて発生が停止し、細胞が白濁して死亡する様子が観察された（図 3B, C）。したがって、媒精 13-14 時間後に計測した胚体形成率はスマ×カツオ雑種およびスマ×マルソウダ雑種では 0%となった（図 1）。一方、スマ、スマ×コシナガ雑種、スマ×クロマグロ雑種の受精卵はエピボリーを完了して、嚢胚を形成した後に、媒精 13-14 時間後に神経胚が形成された（図 2B, I, L）。胚体形成率（mean ± SEM）はスマが 69.5 ± 5.0%（n＝5）、スマ×コシナガ雑種が 44.3%（n＝1）、スマ×クロマグロ雑種が 52.5 ± 21.0%（n＝3）であった（図 1）。さらにスマ、スマ×コシナガ雑種、スマ×クロマグロ雑種はその後も顕著な死亡は確認されず、媒精 30-31 時間後に孵化仔魚が観察された（図 2C, J, M）。孵化率（mean ± SEM）はスマが 64.6 ± 5.2%（n＝5）、スマ×コシナガ雑種が 36.7%（n＝1）、スマ×クロマグロ雑種が 50.3 ± 19.8%（n＝3）であった（図 1）。

図 4 は 1、4、8 日齢のスマ、スマ×コシナガ雑種、スマ×クロマグロ雑種の仔魚を示している。4 日齢のスマ仔魚は目が黒化し開口しており、消化管内には摂餌

されたワムシが観察された（図 4B）。一方、スマ×コシナガ雑種およびスマ×クロマグロ雑種仔魚はスマ仔魚と同様に目が黒化し、開口しているにもかかわらず、ワムシを摂餌しておらず、消化管内にワムシが確認されなかった（図 4E,G）。図 5 はスマ、スマ×コシナガ雑種、スマ×クロマグロ雑種の 1 日齢から 6 日齢の成長を示したグラフである。スマ、スマ×コシナガ雑種、スマ×クロマグロ雑種の孵化直後の全長（mean ± SD）はそれぞれ 3.01 ± 0.05 mm、3.02 ± 0.04 mm、3.04 ±0.05 mm であり有意な差はみられなかった。しかし、スマ×コシナガ雑種およびスマ×クロマグロ雑種は卵黄の吸収が完了する 3 日齢で成長が停止し、6 日齢の全長はスマ×コシナガ雑種が 3.28 ± 0.08 mm （n = 3）、スマ×クロマグロ雑種が 3.17 ± 0.12 mm（n = 3）であり、6 日齢のスマの全長 4.21 ± 0.14 mm（n = 3）と比較して各雑種は有意に小さかった。その後、スマ×コシナガ雑種およびスマ×クロマグロ雑種は 7 日齢で全滅し観察を終了した。

考察

第 3 章ではクロマグロの宿主として利用可能なスマ雑種を作出するために、メスのスマから得た卵に対して 3 属 4 種（カツオ属カツオ、ソウダガツオ属マルソウダ、マグロ属コシナガ、マグロ属クロマグロ）の凍結精子をそれぞれ媒精し、各雑種の初期発生を観察した。その結果、スマ×カツオ雑種、スマ×マルソウダ雑種、スマ×コシナガ雑種、スマ×クロマグロ雑種は全て致死性雑種であることが明らかになった。

クロマグロの宿主として利用するためには、スマ雑種の生残性を回復させる必要がある。一般に、異属間よりは同属間の交雑で生残性雑種が得られる可能性が高い（荒井ら, 2017）。実際に第 1 章で作出したサバ科サバ属の同属間雑種であるゴマサバ×マサバ雑種は、両親種と同等の生残性を示した（Kawamura et al, 2020a）。そこで、スマ属の同属間雑種を作出することができれば、生残性のスマ雑種が得られる可能性がある。サバ科スマ属にはインド太平洋の熱帯・亜熱帯域に分布するスマ、大西洋の熱帯・亜熱帯域に分布するタイセイヨウヤイト *Euthynnus alletteratus*、東太平洋の熱帯・亜熱帯域に分布する Black skipjack *Euthynnus lineatus* の 3 種が分類されており、日本近海に生息している種はスマのみである（Nelson et al., 2016）。すでに航空機で輸送可能なドライシッパーを用いた野外での作業に適した精子凍結技術を第 2 章で開発しており（Kawamura et al., 2020b）、本技術によりサンプリング直後の雄の精子を凍結・運搬することが国外でも可能である。したがって、国外で作製したタイセイヨウヤイトや Black skipjack の凍結精子を当研究室で飼育しているメスのスマから得た卵へ媒精させることでスマ属の同属間雑種を作出可能となる。今後は実際にスマの同属間雑種を作出し、その生残性および妊性を解析する必要がある。

一部のサケ科魚種の致死性雑種は三倍体化処理を施して異質三倍体にすること

で生残性を回復させることが可能である（Scheerer and Thorgaard, 1983; Chevassus et al., 1983; Arai, 1988; Seeb et al., 1988; Gray et al., 1993）。例えば、メスのニジマスとオスのブラウントラウトの交雑によって得られるニジマス×ブラウントラウト雑種は孵化に至らない致死性雑種であるが、この雑種の異質三倍体は正常に孵化し成魚まで生育することが可能である（小原・傳田, 2008）。スマはすでに低温処理による三倍体化技術が確立されており（Yazawa et al., 2019）、今後は本研究によって作出された致死性スマ雑種の生残性を三倍体化により回復できるかどうか検証する必要がある。ただし、三倍体化処理を施しても生残性が回復しない致死性雑種も報告されている。サケ科魚類で報告されている致死性雑種の中には、核型と染色体数が両親種の中間となる二倍体の致死性雑種（シロサケ♀×カワマス♂雑種など）と受精後に染色体が削減されて染色体数が両親の半数和にならない異数体の致死性雑種（イワナ♀×ニジマス♂雑種など）がおり（Arai, 1984; Fujiwara et al., 1997）、後述した異数体の致死性雑種が三倍体化により生残性を回復させた例は知られていない。したがって、異質三倍体が生残性雑種となるかを推測するために致死性スマ雑種の核相解析が必要である。

スマ×コシナガ雑種、スマ×クロマグロ雑種はどちらも胚発生が停止せず孵化に至るが、これらの雑種仔魚はワムシを摂餌せずに死亡することが明かになった。当研究室の長谷川らはゲノム編集技術を用いて作出したアルビノマサバ仔魚が目の黒色素欠損に起因する視覚障害によってワムシを摂餌できないことを明らかにした。このアルビノマサバのワムシ摂餌能を改善する飼育方法としてピンスポット照射飼育法が開発されている（Hasegawa et al., unpublished data）。ピンスポット照射飼育法とは、飼育水槽の上部から白色もしくは緑色の照明を局所的に照射（ピンスポット照射）することで、仔魚とワムシの正の走行性により明部へ仔魚とワムシを蝟集させ、仔魚の周囲に局所的なワムシの超高密度環境を形成させる飼育法である。本法により、視覚が弱いアルビノマサバでもワムシを摂餌することが可能になった。

したがって、ワムシを摂餌せずに死亡したスマ×コシナガ雑種、スマ×クロマグロ雑種をピンスポット照射飼育法で飼育することでワムシ摂餌能の改善が期待される。

スマ×カツオ雑種およびスマ×マルソウダ雑種では、胞胚期からエピボリー期で発生が停止し、死亡することが明かになった。魚類の胚発生において、卵割の時期は母性因子によって制御されているが、中期胞胚遷移以降は母性因子が分解され始め、胚性因子による制御へと置き換わる（Tadros and Lipshitz, 2009）。したがって、致死性雑種の死亡時期は胚ゲノムの遺伝子発現が開始する胞胚期以降、特に嚢胚期や胚体頭部形成の時期、および循環系形成期であることが多い（荒井ら, 2017）。スマ×カツオ雑種およびスマ×マルソウダ雑種の発生が停止した胞胚期～エピボリー期は中期胞胚遷移の時期であるため、両雑種の雑種致死の原因は胚性因子の発現不全によるものと推測された。

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図説明

図 1. スマおよび各スマ雑種における初期生残率の平均値

交雑試験によって得られたスマ（$n=5$）、スマ×カツオ（$n=2$）、スマ×マルソウダ（$n=2$）、スマ×コシナガ（$n=1$）、スマ×クロマグロ（$n=3$）の受精率、胚体形成率、孵化率の平均値のグラフである。データは平均値 ± 標準誤差で示している。

図 2. スマおよび各スマ雑種の受精から孵化まで

図は上段からそれぞれスマ（A-C）、スマ×カツオ雑種（D, E）、スマ×マルソウダ雑種（F, G）、スマ×コシナガ雑種（H-J）、スマ×クロマグロ雑種（K-M）の 4 細胞期（左図；A, D, F, H, K）、胚体形成（中央図；B, E, G, I, L）、孵化仔魚（右図；C, J, M）を示している。4 細胞期は媒精 1-2 時間後に、胚体形成は媒精 13-14 時間後に、孵化仔魚は媒精 30-31 時間後に観察した。ただし、スマ×カツオ雑種およびスマ×マルソウダ雑種の発生がエピボリーで停止して胚体形成卵が得られなかったため、E,G は媒精 8-9 時間後に観察した卵を示している。また、スマ×クロマグロ雑種の孵化仔魚（M）は媒精 48 時間後に観察した。

図 3. 媒精 8-9 時間後のスマ（A）、スマ×カツオ雑種（B）、スマ×マルソウダ雑種（C）の受精卵

スケールバーは 500 μm を示している。

図 4. 1、4、8 日齢のスマ（A-C）および 1、4 日齢のスマ×コシナガ雑種（D, E）、スマ×クロマグロ雑種（F, G）

図は上段からそれぞれスマ（A-C）、スマ×コシナガ雑種（D-E）、スマ×クロマグロ雑種（F-G）の 1 日齢（左図；A, D, F）、4 日齢（中央図；B, E, G）、 8 日齢（右図；

C）の仔魚であり、矢じりは消化管内の摂餌されたワムシを示している。スケールバーは 500 μm を示している。

図 5. スマ、スマ×コシナガ雑種、スマ×クロマグロ雑種の 1 日齢から 6 日齢までの成長

各プロットは 3 個体の全長の平均値±標準偏差を示している。アスタリスクはスマの全長と有意な差があることを示している（$p < 0.05$）

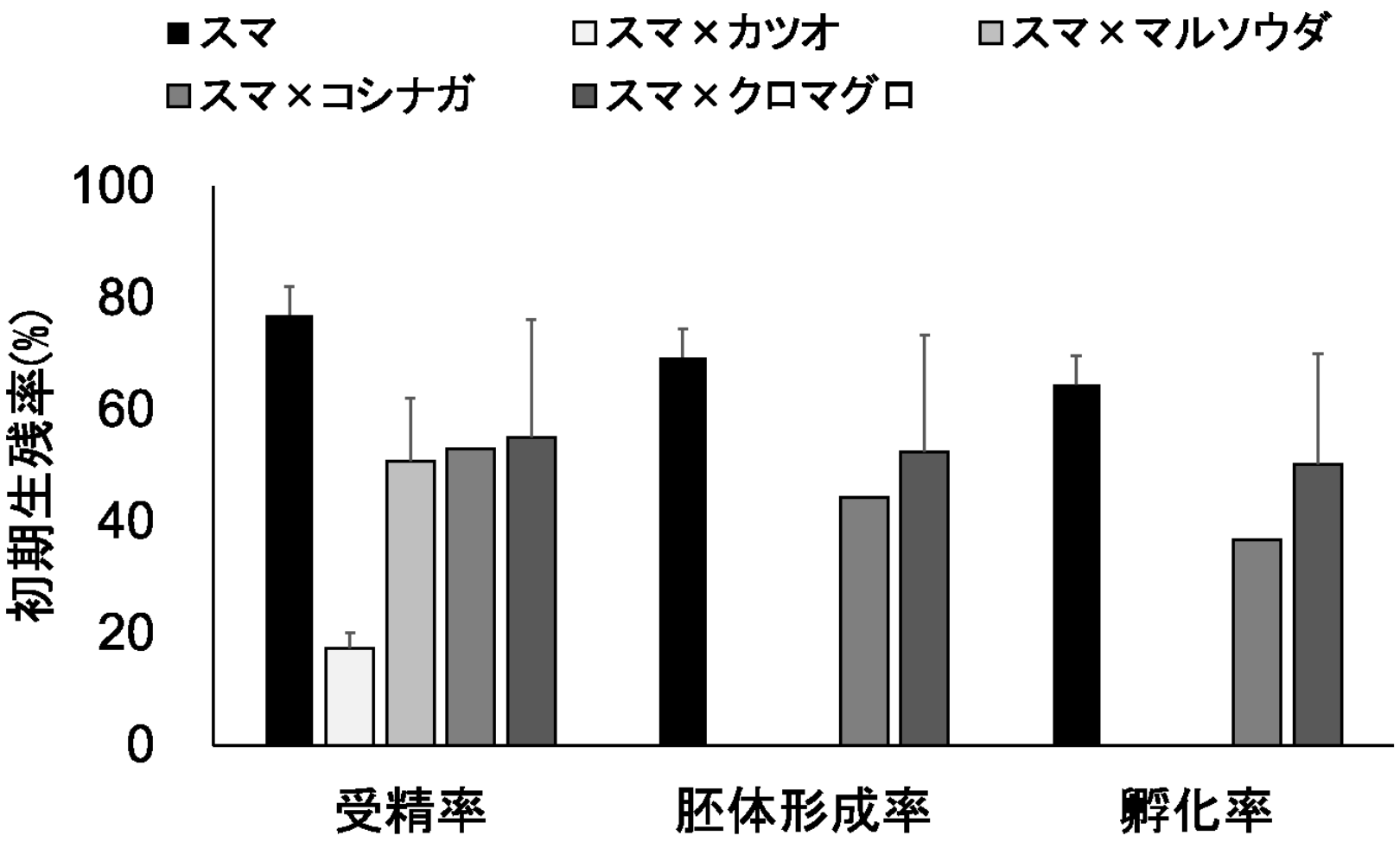

図1. スマおよび各スマ雑種における初期生残率の平均値

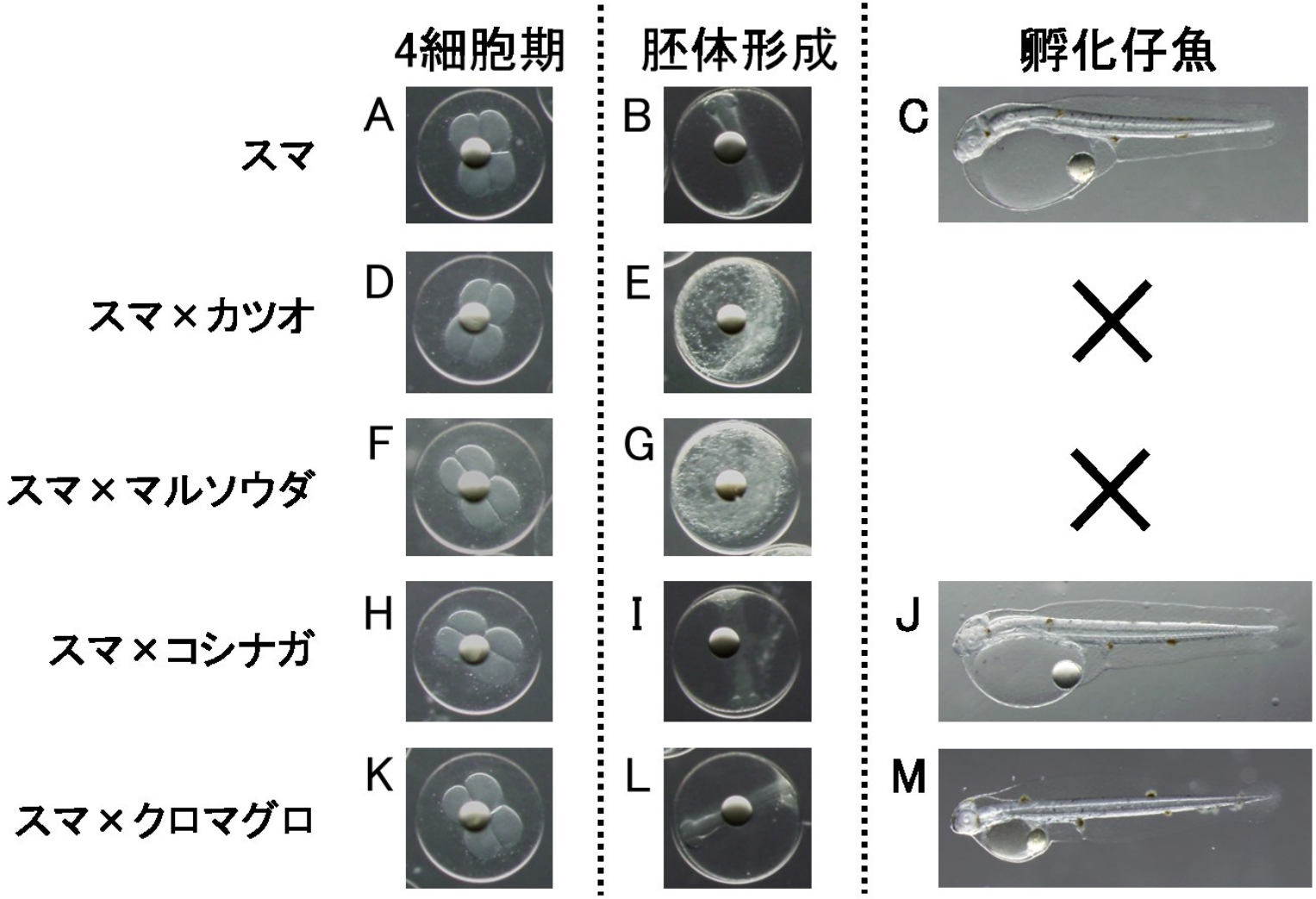

図2. スマおよび各スマ雑種の受精から孵化まで

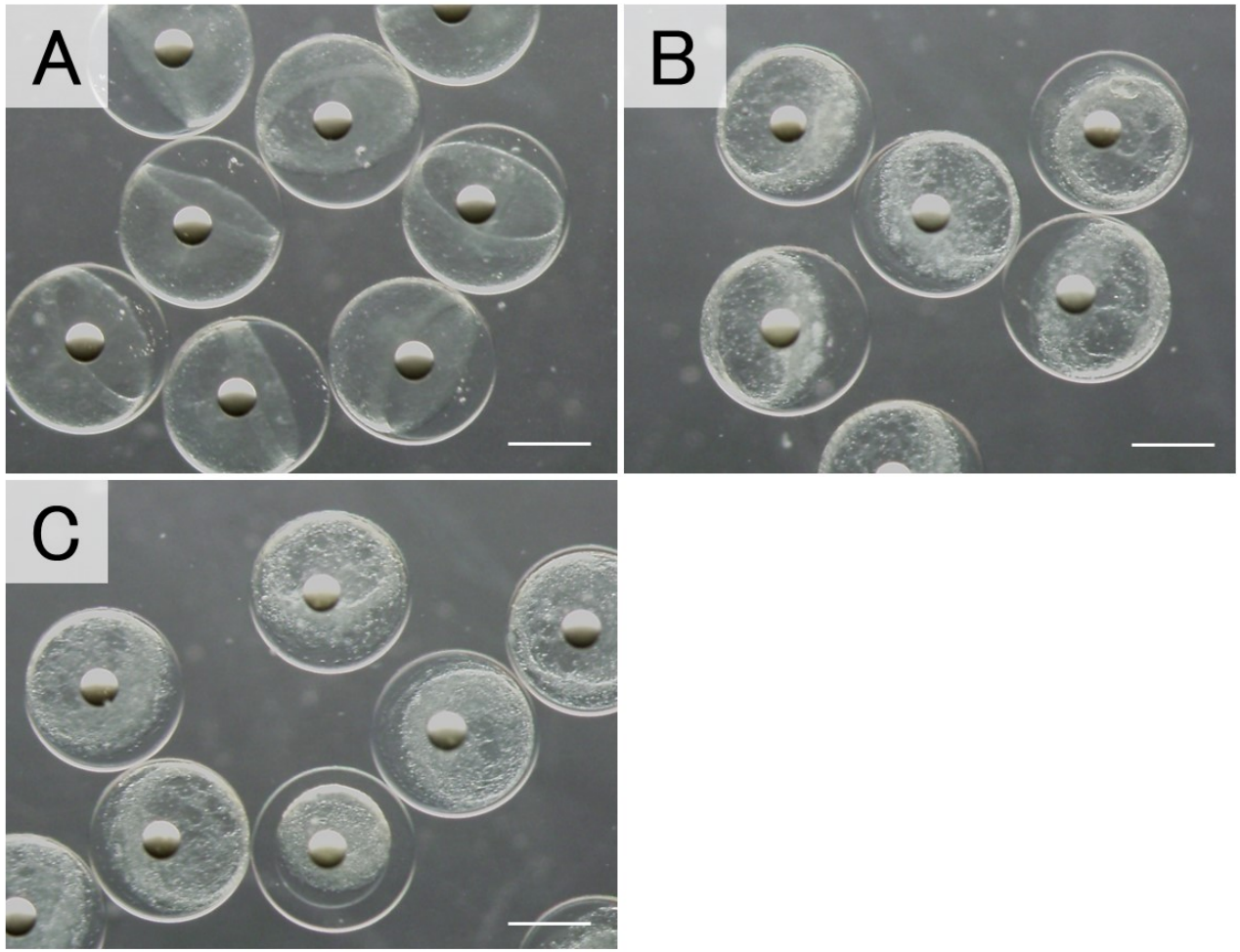

図3. 媒精8–9時間後のスマ（A）、スマ×カツオ雑種（B）、
スマ×マルソウダ雑種（C）の受精卵

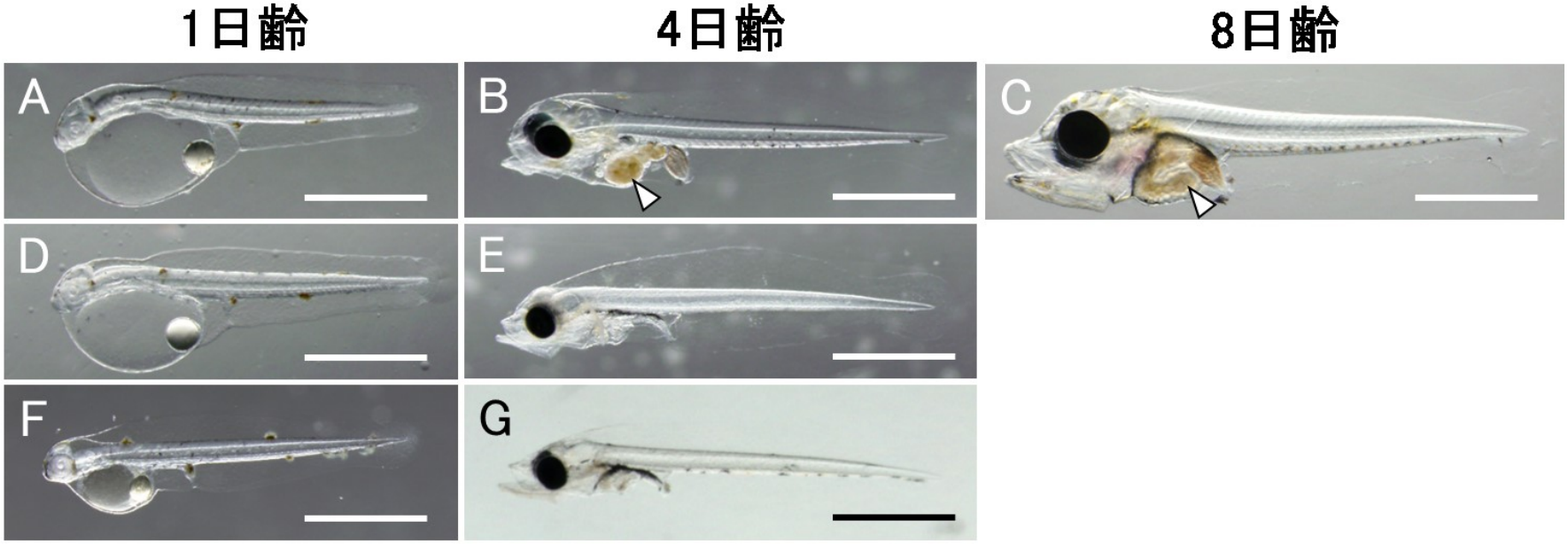

図4. 1、4、8日齢のスマ(A-C)および
1、4日齢のスマ×コシナガ雑種(D, E)、スマ×クロマグロ雑種(F, G)

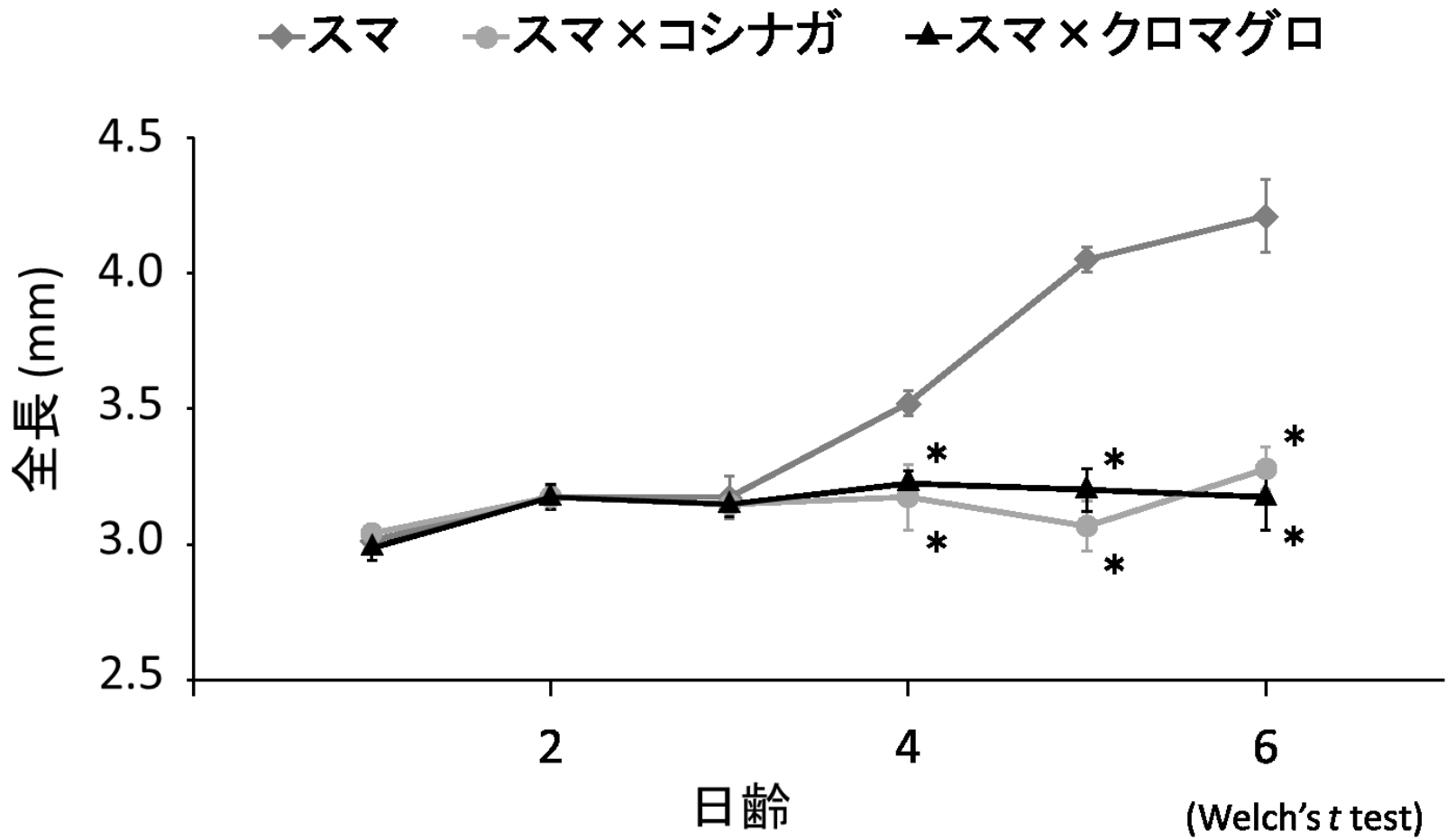

図5.スマ、スマ×コシナガ雑種、スマ×クロマグロ雑種の1日齢から6日齢までの成長

表1. 交雑試験の初期生残率

日付 年	月	日	オス親魚種	全卵数 (粒)	浮上卵率[*1] (%)	浮上卵数 (粒)	受精率[*2] (%)	胚体形成率[*2] (%)	孵化率[*2] (%)
2013	10	24	スマ	3450	94.2	3250	83.8	76.8	74.0
			クロマグロ	26000	100	26000	84.5	80.9	77.5
2013	10	31	スマ	10660	100	10660	80.6	77.9	77.9
			クロマグロ	34660	100	34660	14.0	11.6	11.6
2013	11	15	スマ	13440	99.2	13333	90.0	78.3	65.0
			クロマグロ	24880	96.5	24000	66.7	65.0	61.7
2015	10	4	スマ	13333	97.2	12963	68.1	56.9	52.7
			カツオ	65333	96.9	63288	14.6	0	0
			マルソウダ	47667	96.1	45829	62.1	0	0
2015	10	8	スマ	38000	69.1	26258	62.8	57.4	53.3
			カツオ	35000	78.8	27575	20.1	0	0
			マルソウダ	35667	87.6	31230	39.6	0	0
			コシナガ	60667	79.9	48453	53.0	44.3	36.7

＊1；全卵数に対する割合を示す

＊2；浮上卵数に対する割合を示す

総括

本研究ではクロマグロの代理親として利用可能なサバ科不妊宿主の作出を目指し、サバおよびスマの種間雑種の作出とその特性解析を行った。第 1 章ではゴマサバ♀とマサバ♂の種間交雑により生殖細胞欠損不妊を示すゴマサバ×マサバ雑種の作出に成功した。サケ科魚類の先行研究では、生殖細胞欠損不妊宿主は三倍体宿主（減数分裂異常により不妊化するため生殖細胞自体は存在する）よりも異種由来配偶子の生産効率が高いことが明かになっている（Yoshizaki et al., unpublished data）。これは、生殖細胞欠損宿主が自身の生殖細胞を保持しないために、移植されたドナー生殖細胞が空の生殖細胞ニッチを占有し、効率的に増殖・分化できたためであると考えられる。実際に移植後細胞の挙動を追跡した解析では、二倍体宿主よりも生殖細胞欠損不妊宿主はドナー由来生殖細胞の生着および増殖を効率的に支持可能であることが証明されている（Yoshizaki et al., 2016）。したがって、生殖細胞欠損不妊であるゴマサバ×マサバ雑種はクロマグロ配偶子の生産に極めて有利であると考えられる。さらに、ゴマサバ×マサバ雑種仔魚へクロマグロ精巣細胞を移植したところ、ゴマサバ×マサバ雑種の生殖腺はクロマグロの精原細胞を高効率（100.0%）で取り込み可能であることが明らかになった。また、ゴマサバ×マサバ雑種は人工授精のみの簡便な操作で大量生産が可能であり、成魚まで問題なく生育することを明らかにした。以上の結果からゴマサバ×マサバ雑種は宿主として理想的な生殖細胞欠損不妊を示し、クロマグロ精原細胞の取り込み効率が高く、人工授精のみで大量生産が可能であることから、本雑種はクロマグロ代理親として極めて有望であることが明かになった。今後はゴマサバ×マサバ雑種を宿主としてクロマグロ配偶子の生産を目指す。

以上のように、初期減耗の著しいサバ科魚種においても種間交雑によって不妊宿主を大量生産可能であることが第 1 章で実証された。そこで本研究では

当研究室でかねてよりクロマグロの宿主候補として期待されていたスマの種間交雑にも着手した。一般に、宿主種とドナー種が遺伝的に近縁であるほど宿主のドナー由来配偶子の生産効率が高い（Bar et al., 2016; Yoshizaki and Yazawa, 2019; Goto and Saito, 2019）。したがって、サバよりもクロマグロと遺伝的に近縁であり、産卵適水温等の生理学的な性質もクロマグロに類似しているスマの不妊雑種が作出できれば、このスマ雑種はクロマグロ配偶子を高効率で生産する宿主になることが期待される。しかし、回遊魚であるスマ近縁種の凍結精子を作製するためには、波で揺れる船上や強い海風が吹く漁港といった不安定な野外環境での作業が必要となる。そこで、第 2 章では野外でも簡便に作業が可能なドライシッパーを用いた凍結精子作製法を開発した。本法を用いることによってスマ近縁種のカツオ属カツオ、ソウダガツオ属マルソウダ、マグロ属クロマグロ、マグロ属コシナガの凍結精子を作製することに成功した。

第 3 章では、メスのスマ親魚から得た卵と第 2 章で作製した 3 属 4 種（カツオ、マルソウダ、コシナガ、クロマグロ）の凍結精子を交雑し、各雑種の初期発生を観察した。その結果、スマ×カツオ雑種、スマ×マルソウダ雑種、スマ×コシナガ雑種、スマ×クロマグロ雑種は全て致死性雑種であることが明らかになった。一般に、異属間よりは同属間の交雑で生残性雑種が得られる可能性が高い（荒井ら, 2017）。実際に、第 1 章で作出したサバ科サバ属の同属間雑種であるゴマサバ×マサバ雑種は生残性の雑種である。そこで今後は大西洋の熱帯・亜熱帯域に生息するスマ属タイセイヨウヤイトおよび東太平洋の熱帯・亜熱帯域に生息する Black skipjack の凍結精子を作製し、スマ属同士の同属間雑種を作出する予定である。これらの凍結精子の作製は国外かつ野外での作業となるが、第 2 章で開発したドライシッパーを用いた精子凍結法により、少ない労力で精子の凍結および運搬が可能である。同属間の交雑によって生残性のスマ雑種を作出することができれば、クロマグロ配偶子を生産するための有力な宿主候補になると期待される。

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