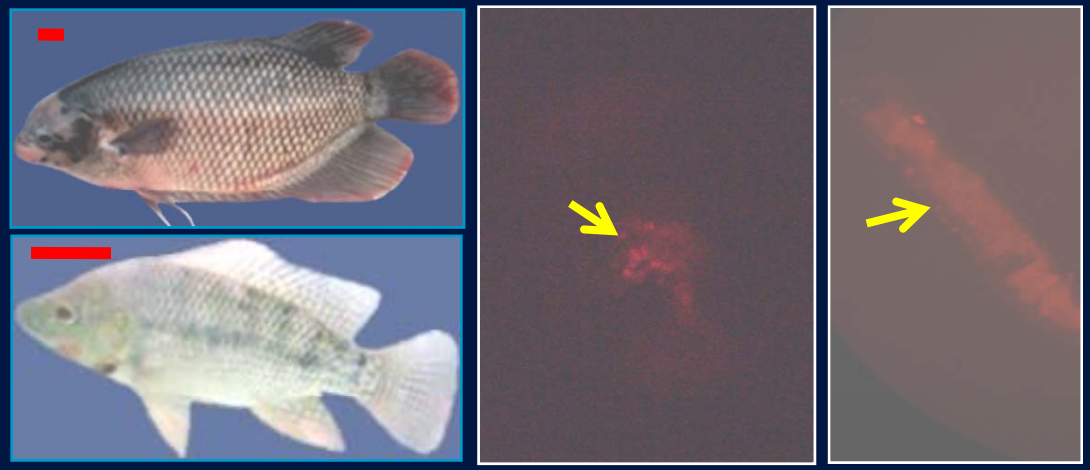
TEKNOLOGI TRANSPLANTASI SEL TESTIKULAR DALAM REKAYASA PRODUKSI BENIH IKAN GURAME (*Osphronemus gouramy*)

Testicular cell transplantation technology in manipulation of giant gouramy fry production



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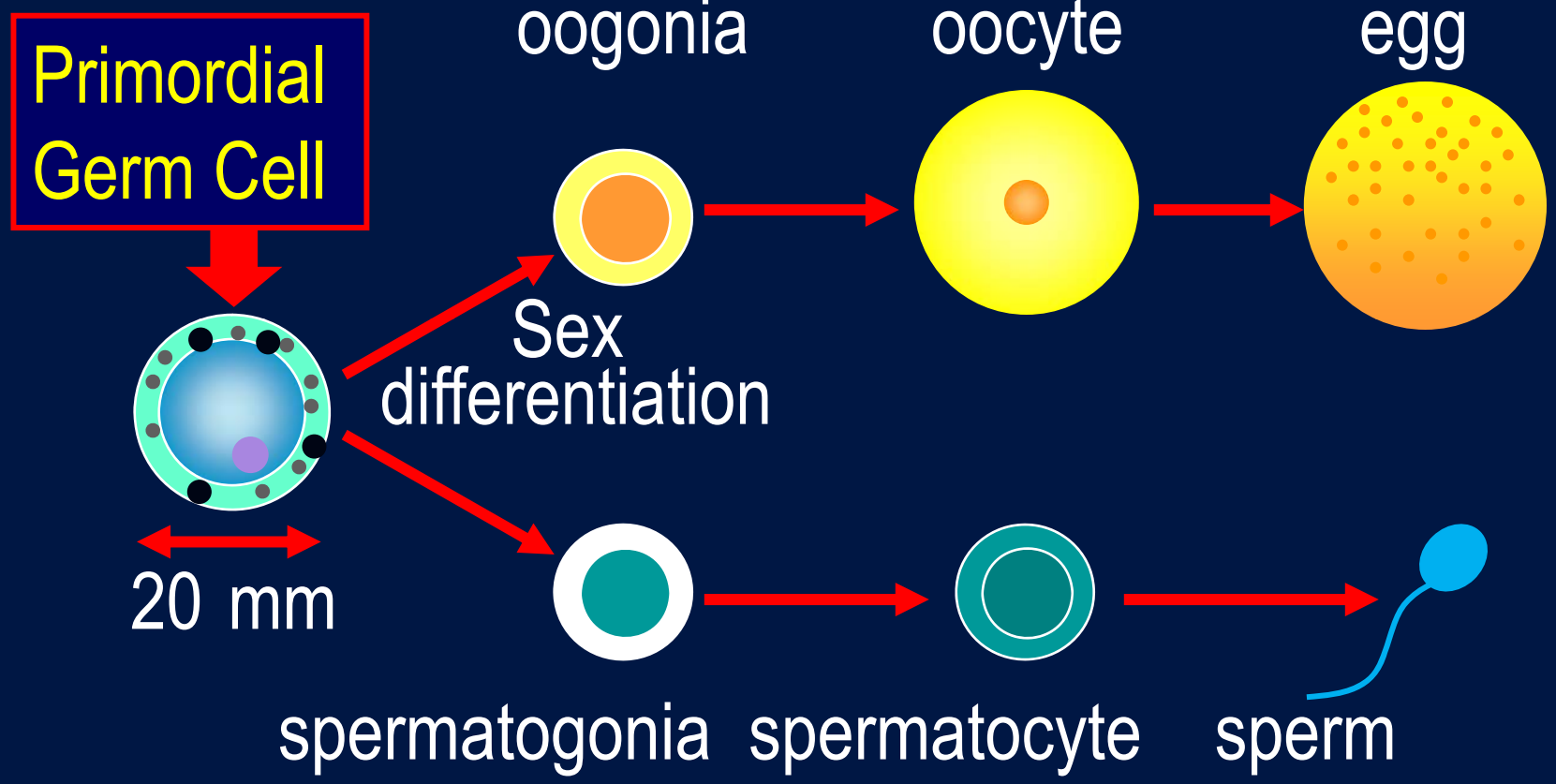
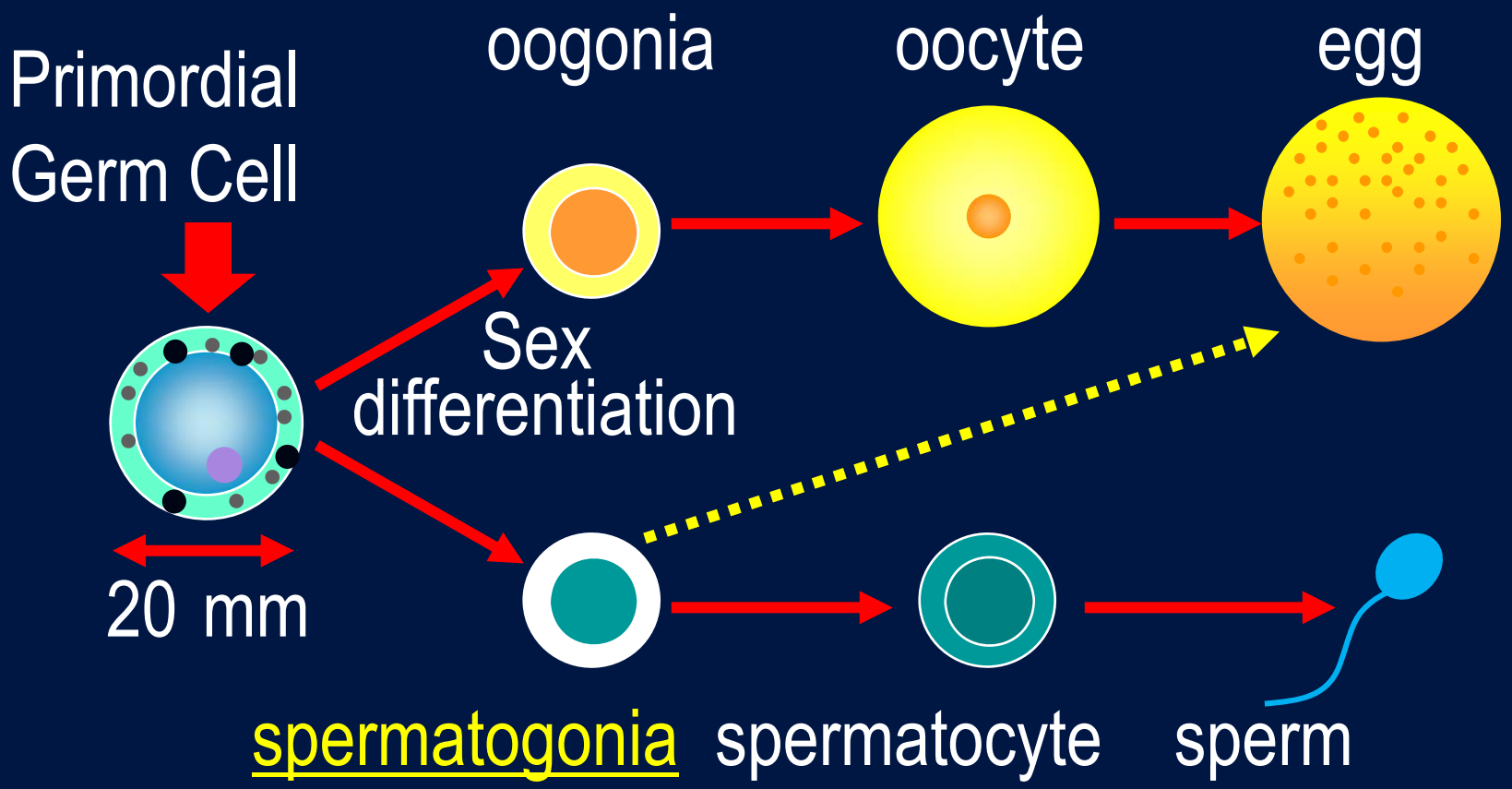
Rainbow trout juveniles produced by surrogated masu salmon

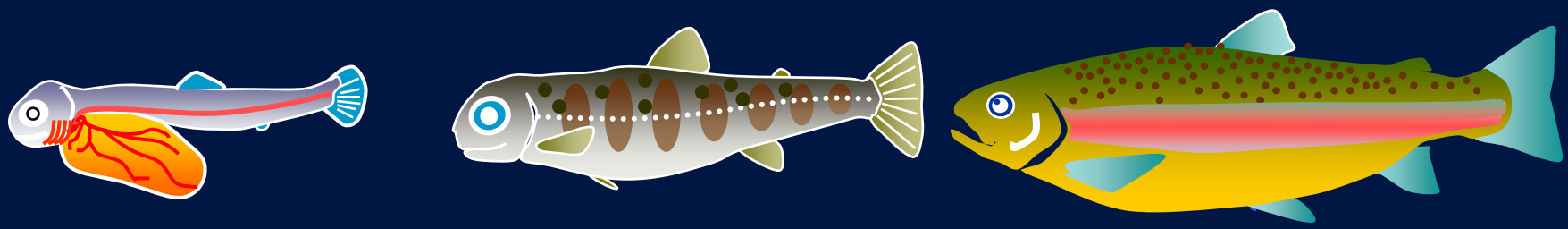
Donor-derived offspring from inter-species germ-cell transplantation were successfully produced (Takeuchi *et al*. 2004, Nature)

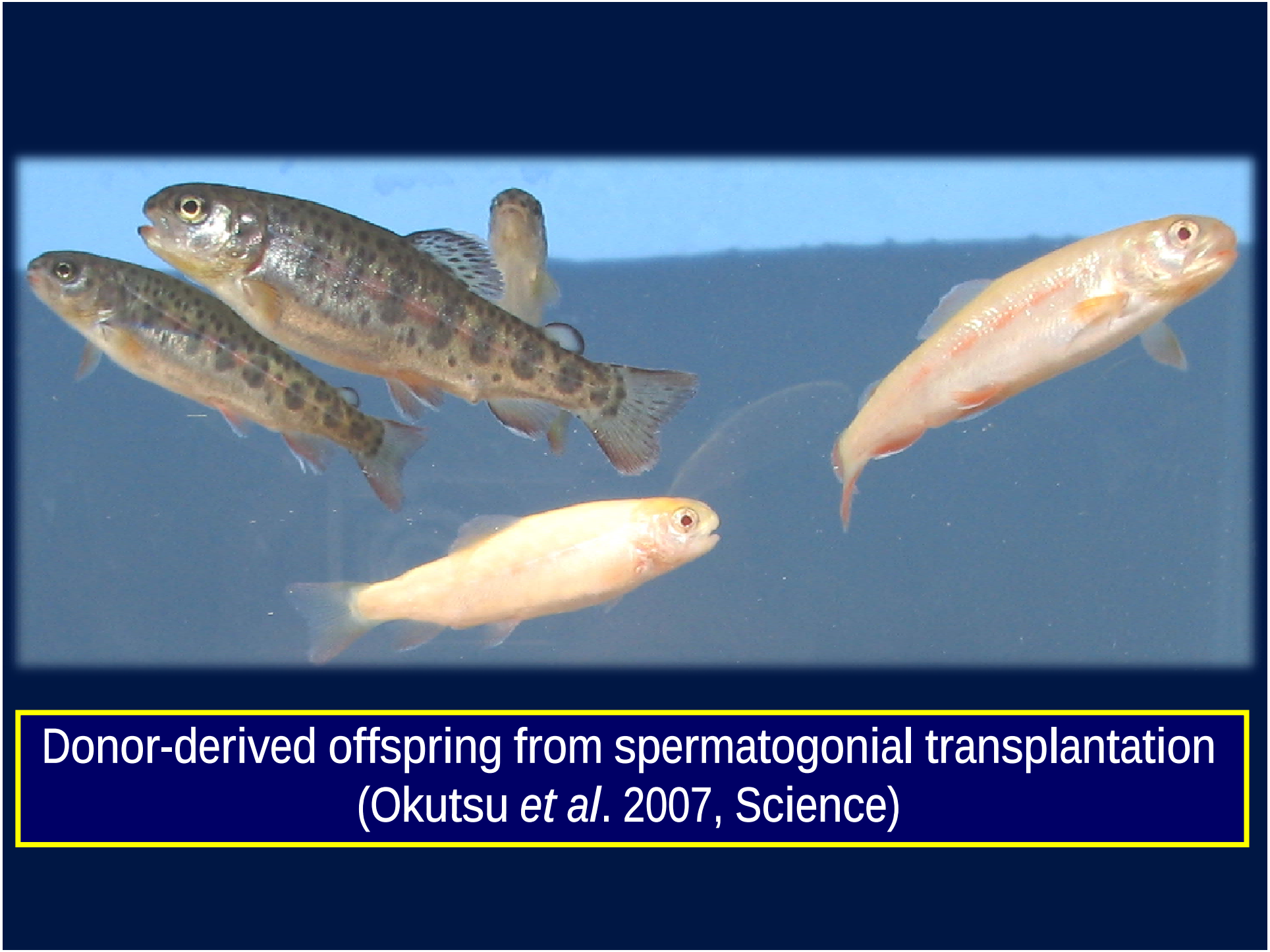
Primordial Germ Cells



Spermatogonial stem cells



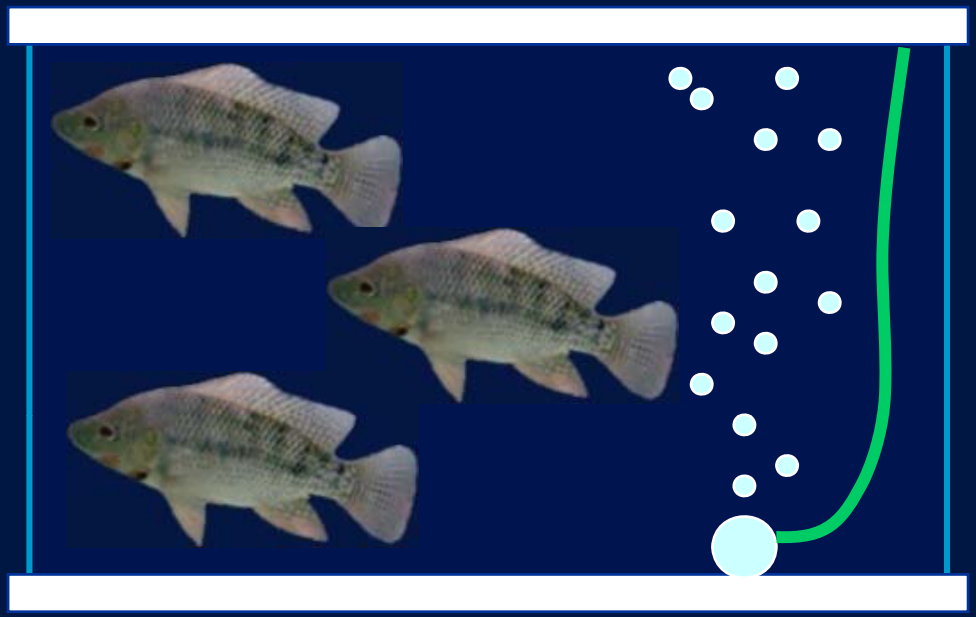


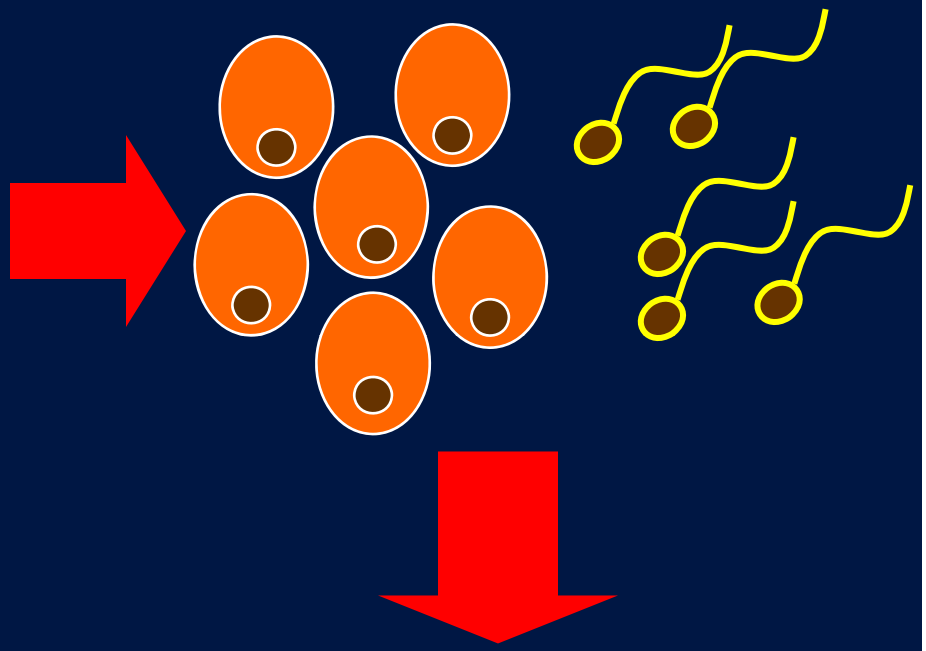


Donor-derived offspring from spermatogonial transplantation
(Okutsu *et al*. 2007, Science)

	Giant gouramy	Nile tilapia
Mature size;	2 - 4 kg	200 - 400 g
Time to get mature;	3 - 4 years	4 - 6 months
Spawning;	Natural, pond	Semi/artificial aquarium

Nile tilapia as surrogated broodstock





Egg can be produced faster

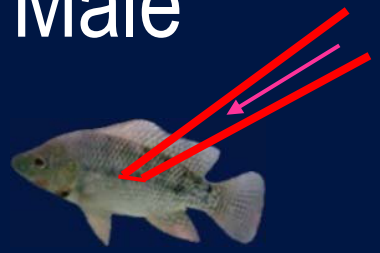
* save a lot of time, labor and cost.

* environmental manipulation and genetic engineering can be easily performed.

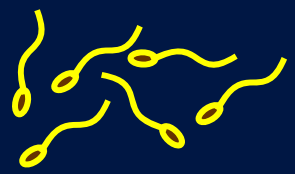
Final goal of research:

Spermatogonia

Male

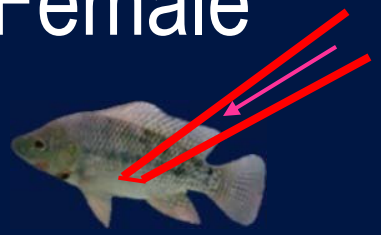


Donor sperm



recipients

Female



Donor egg

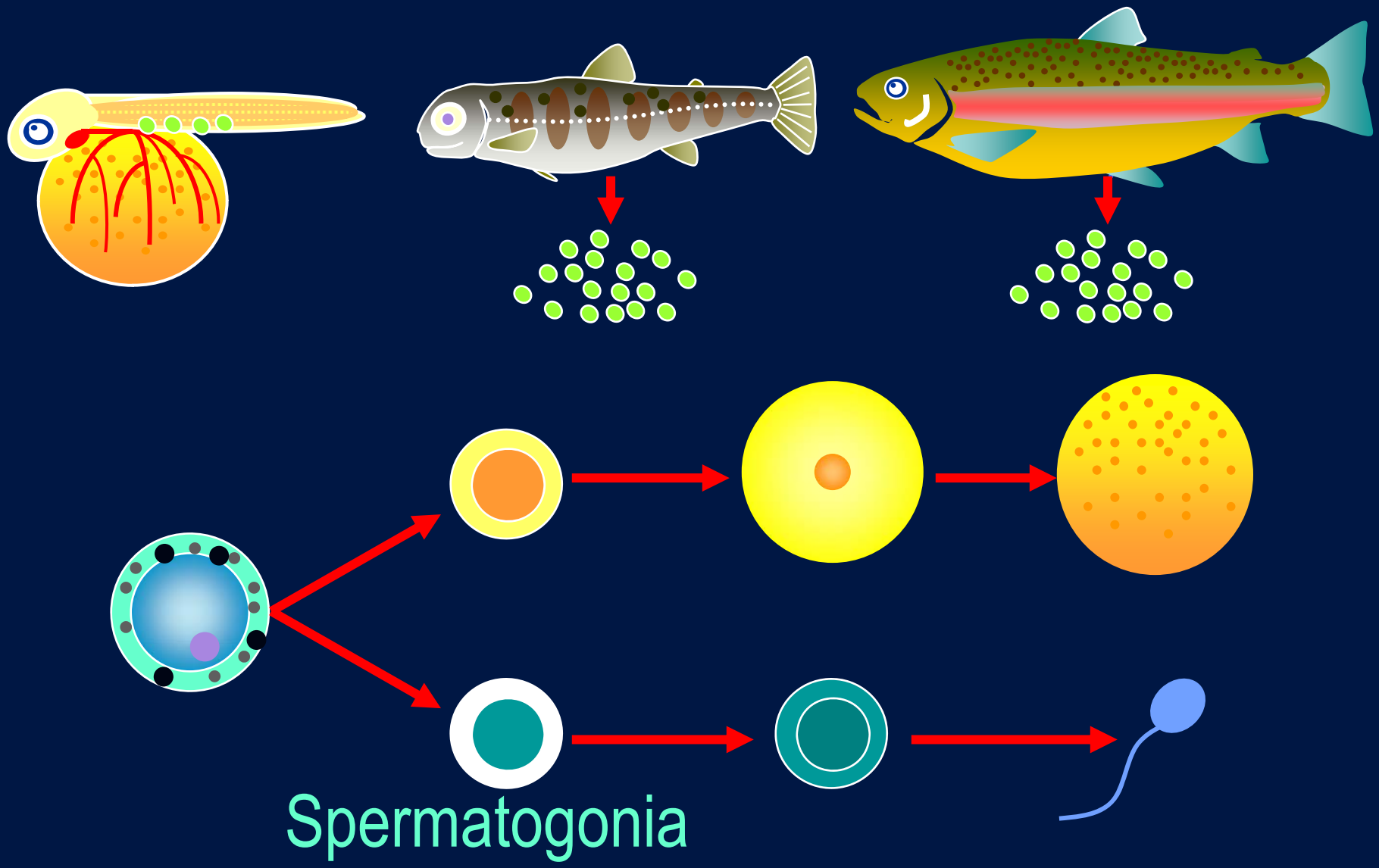
Steps of study (First Year):

1. Characterization of testicular germ cells in giant gouramy
2. Establishment of testicular germ cells dissociation method
3. Analysis of donor testicular germ cells colonization in recipient gonad

Supporting research (First Year):

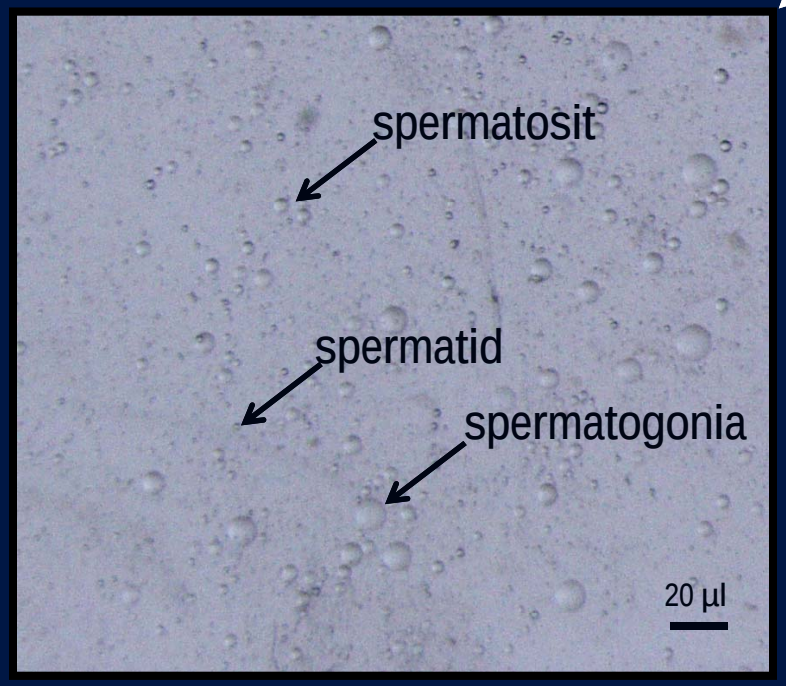
1. cDNA cloning and expression analysis of *vasa-like* gene in giant gouramy
2. Development of DNA molecular marker in gonadal cell identification by PCR method

1. Characterization of testicular germ cells in giant gouramy

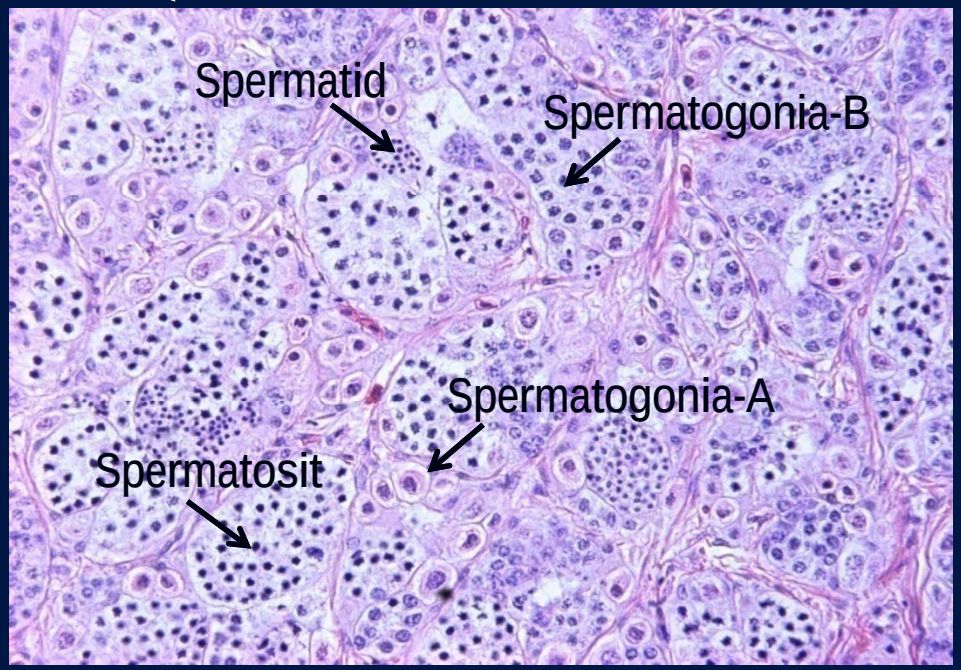


Ukuran ikan gurame yang banyak mengandung spermatogonia ?

Testis dari ikan gurame ukuran 800 g



Sel testikular hasil disosiasi



Histologi testis, pewarna HE

Hasil penelitian:

Jumlah dan persentase sel spermatogonia pada berbagai ukuran berat tubuh ikan gurame

No.	Berat ikan (g)	Berat gonad (g)	GSI ($x10^{-3}$%)	Jumlah dan persentase spermatogonia
1.	530	0,04	7,55	990.000 (63,87)
2.	920	0,07	7,61	385.000 (23,91)
3.	1100	0,09	8,18	258.750 (12,97)
4.	1300	0,11	8,46	264.000 (12,63)
5.	1600	0,15	9,38	187.500 (10,15)
6.	2300	0,23	9,57	149.500 (6,02)

GSI (gonado somatic index) : (berat gonad / bobot tubuh ikan) x 100%
Larutan disosiasi : trypsin 0,5% dalam PBS

Kesimpulan:

Jumlah dan persentase sel spermatogonia menurun dengan meningkatnya ukuran berat tubuh ikan gurame.

2. Establishment of testicular gem cell dissociation method

Tujuan: diperoleh banyak sel spermatogonia dan viabel/hidup

Perlakuan:

- Dua jenis larutan disosiasi:
    1. Trypsin-phosphate buffer saline (Tryp-PBS)
    2. Trypsin-phosphate buffer saline/fetal bovine serum/DNase I (Tryp-PBS/FBS/DNase)
- Lama inkubasi: 1, 2, 3, 4 dan 5 jam,
- Suhu ruang,
- Trypan blue.

Sel testikular ikan gurame hasil disosiasi. Tanda panah garis penuh: sel yang viabel; tanda panah garis putus: sel yang mati.

Jumlah dan viabilitas sel testikular ikan gurame hasil disosiasi.

Larutan disosiasi	Lama inkubasi (jam)	Jumlah sel	Viabilitas sel (%)
Tryp-PBS	1	515.555 ± 134.219	100,00 ± 0,00
	2	662.222 ± 174.016	96,77 ± 3,23
	3	573.334 ± 300.222	91,87 ± 8,66
	4	577.778 ± 84.678	93,70 ± 2,07
	5	680.000 ± 18.856	90,79 ± 1,86
Tryp-PBS/ FBS/DNase	1	302.222 ± 131.543	100,00 ± 0,00
	2	484.445 ± 180.041	98.24 ± 3,04
	3	568.889 ± 250.274	94,95 ± 5,28
	4	471.111 ± 116.492	95,29 ± 5,74
	5	1.520.000 ± 207.846	87,84 ± 1,74

Kesimpulan:

- Kedua larutan disosiasi bisa digunakan
- Lama inkubasi ≤ 2 jam

3. Analysis of donor cells colonization in recipient gonad

Tujuan:

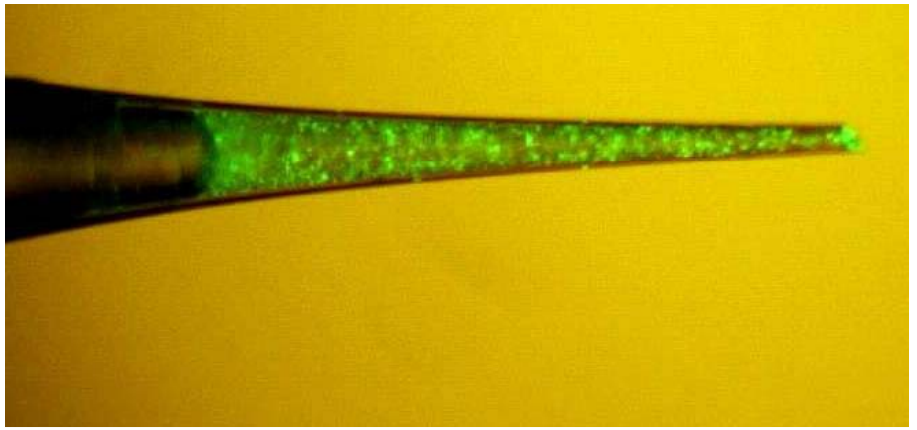
- Sel donor dapat terkolonisasi dalam individu resipien?
- Umur resipien saat transplantasi mempengaruhi persentase kolonisasi sel donor?
- Sel donor hasil disosiasi menggunakan kedua larutan disosiasi dapat terkolonisasi?

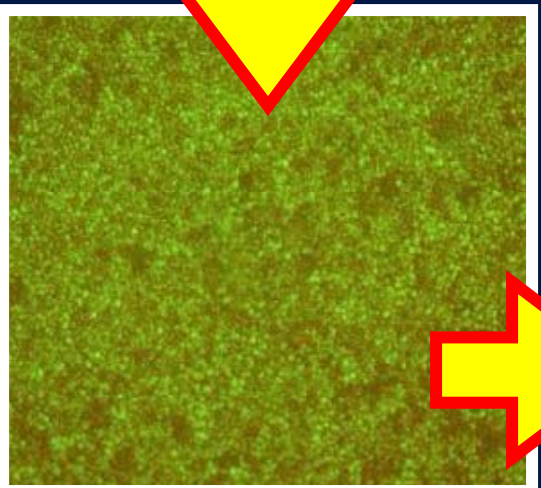
Tahapan:

1. Pengembangan metode identifikasi sel donor dalam individu ikan resipien
2. Transplantasi sel donor dan pemeliharaan ikan hasil transplantasi
3. Analisis kolonisasi

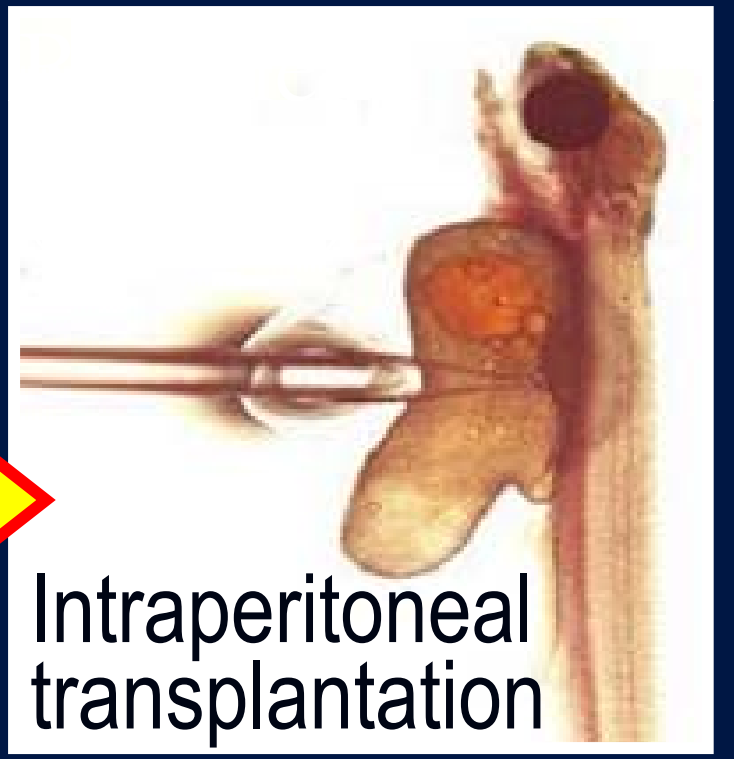
3.1. Metode identifikasi sel donor dalam individu resipien:

1. Sel donor berlabel gen berpendar, *green fluorescent protein* (GFP); ikan transgenik
2. Injeksi mRNA GFP-vasa
3. Sel dilabel PKH26 (sel berwarna merah)
4. PCR, metode alternatif bila warna PKH26 hilang setelah ikan matang gonad





Testicular cell suspension



3.1. Metode identifikasi sel donor dalam individu resipien:

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2. Injeksi mRNA GFP-vasa

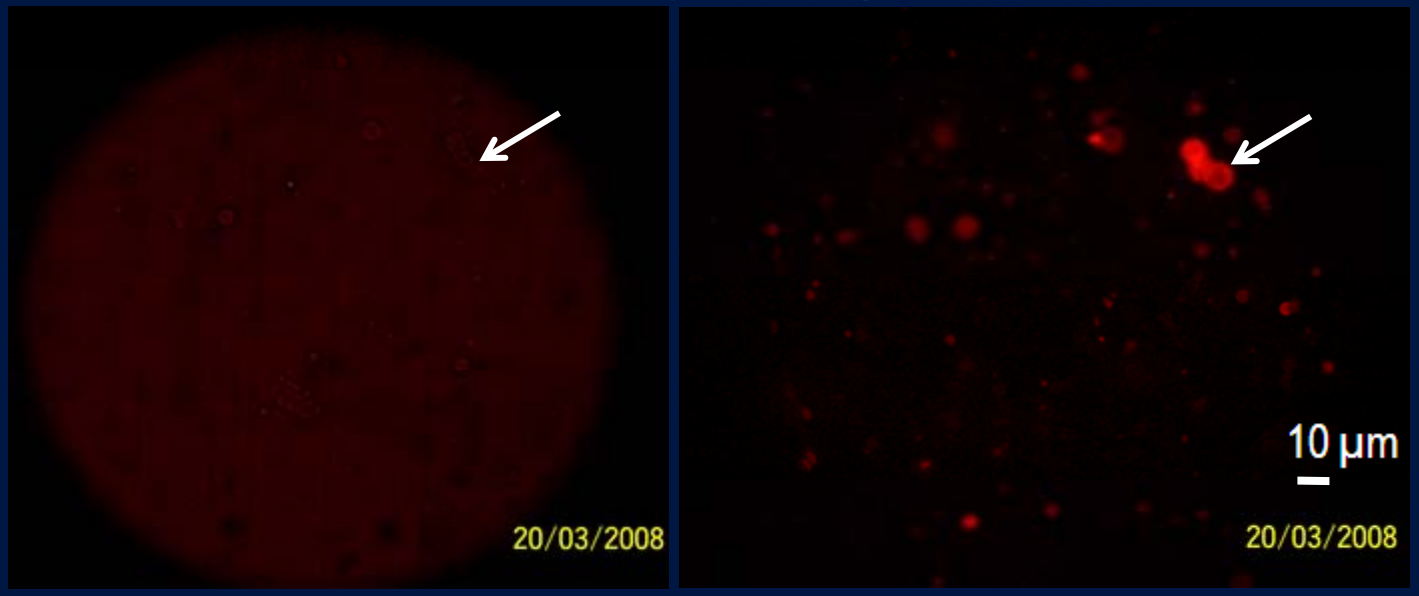
√ 3. Sel dilabel PKH26 (sel berwarna merah)

√ 4. PCR, metode alternatif bila warna PKH26 hilang setelah ikan matang gonad

Cell labeling using PKH26 (Sigma):

Disosiasi sel testikular : tripsin 0,5% dalam PBS selama 2 jam

Dosis PKH26 : 2 µl / 200 µl

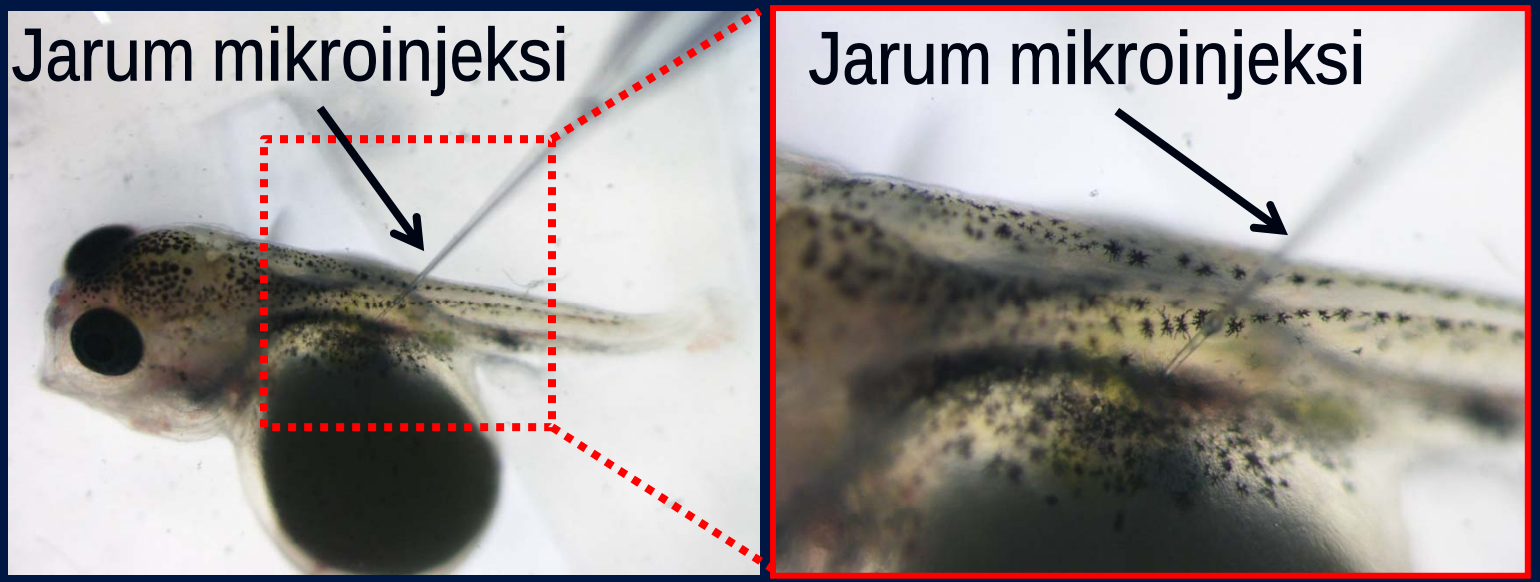


Lama inkubasi (menit)	Persentase sel berlabel (%)
5	28,57
10	50,00
20	81,25

√

3.2. Transplantasi

1. Umur resipien *vs*. kelangsungan hidup benih ikan nila hasil transplantasi
2. Sel donor hasil disosiasi dengan larutan berbeda *vs*. kelangsungan hidup benih ikan hasil transplantasi, dan
3. Persentase kolonisasi



Kelangsungan hidup benih ikan nila hasil transplantasi dan kontrol

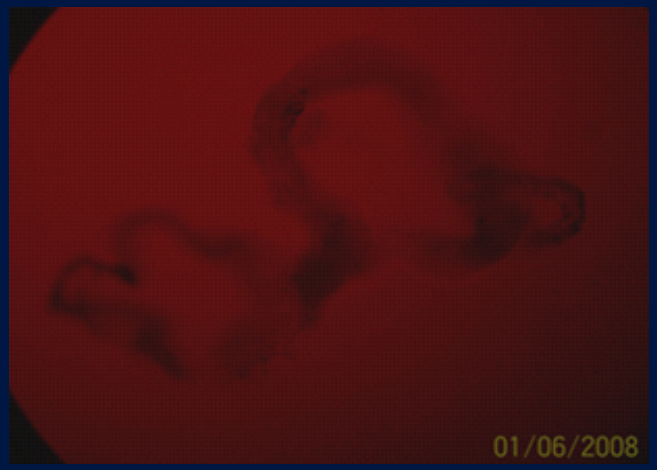
Metode disosiasi	Umur larva (hari)	Jumlah ikan awal (ekor)	Jumlah ikan akhir*) (ekor)	SR (%)
Tryp-PBS	2	17	11	64,71
	3	20	12	60,00
		7	5	71,43
	5	10	6	60,00
Tryp-PBS/FBS/DNase	1	15	10	66.67
	2	15	8	53,33
		20	12	60,00
	3	10	6	60,00
		10	6	60,00
		20	13	65,00
	4	21	14	66,67
	5	12	9	75,00
Kontrol	1	15	10	66.67
	2	20	13	65,00
	3	10	4	40,00
	4	20	20	100,00
	5	20	14	70,00

- Kelangsungan hidup ikan hasil transplantasi relatif sama

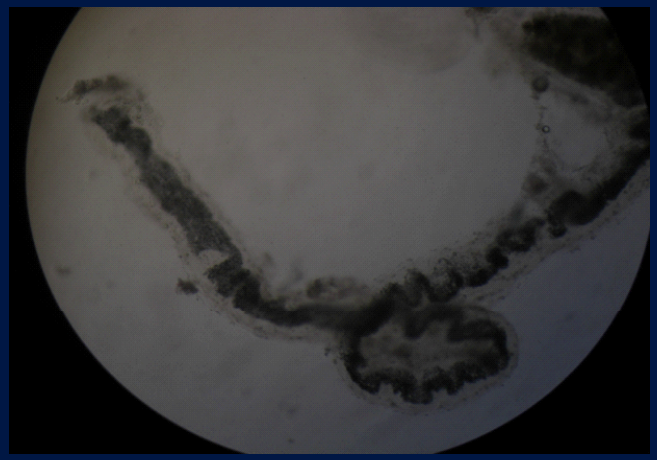
3.3. Analisis kolonisasi sel donor dalam individu resipien

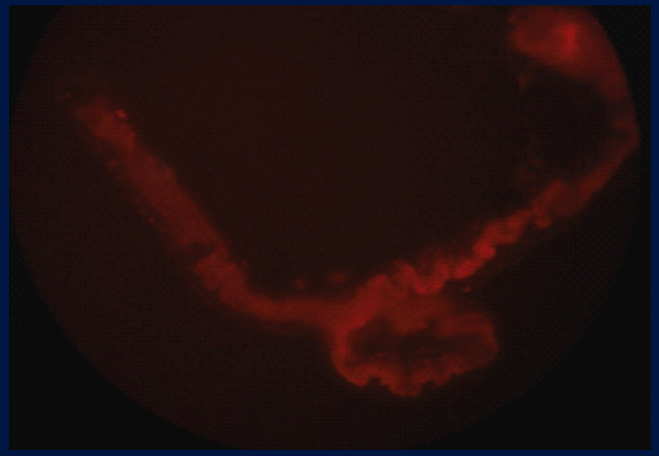
Tanpa filter

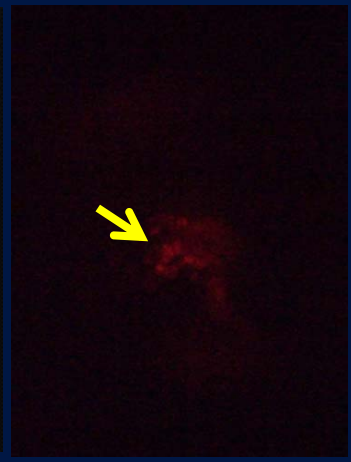
Filter merah



Kontrol







Persentase kolonisasi sel donor ikan gurame dalam gonad ikan nila

Metode disosiasi	Umur larva (hari)	Jumlah ikan diperiksa (ekor)	Persentase kolonisasi	Estimasi jumlah sel terkolonisasi
Tryp-PBS	2	4	25	38
	3	NA	NA	NA
	5	4	25	6
Tryp-PBS/ FBS/DNase	1	NA	NA	NA
	2	4	50	28 ; 55
		4	75	47; 50; 91
	3	NA	NA	NA
	4	NA	NA	NA
	5	2	100	banyak

NA: belum dianalisis

Kesimpulan:

- Sel spermatogonial ikan gurame dapat terkolonisasi dalam gonad ikan nila
- Kedua larutan disosiasi dapat digunakan dalam disosiasi sel testikular untuk transplantasi

Penelitian Lainnya:

1. Kloning dan karakterisasi gen *vasa* ikan gurame
2. Pengembangan marka molekular untuk identifikasi sel donor

1. cDNA cloning and expression analysis of *vasa-like* gene in giant gouramy (*Osphronemus gouramy*)

- RT-PCR method
- Sequencing at Tokyo University of Marine Science & Technology
- Total length of GgVLG cDNA: 2340 bp
- GenBank accession no. : GQ422440 (July 30, 2009)

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- Amino acid residues:

```
MDEWEEEETTTISTIALTSQSTNEGTQGDFWKPDSGESGRGRGGGGRGRRGGFKSSFSSG    60

GEERRDDGNNWNSTAAERGGFRGRGGRGRGRGFGRMDQSEFNGDDSGVCESGFRGGSRGG   120

RGSRGRGGFREAGDQGGRGGYGGGYRGKDEEIFAQGENKDPGKKDAIDGDRPKVTYVPPT   180

LPEDEDSIFAHYKTGINFDKYDDIMVDVSGTNPPQAILTFDEAALCETLRKNVSKSGYVK   240

PTPVQKHGIPIISAGRDLMACAQTGSGKTAAFLLPILQQLMADGVAASRFSELQEPEALI   300
                                ATP-A
VAPTRELINQIYLEARKFSFGTCVRPVVVYGGVSTAHQIREISRGCNVLCGTPGRLLDVI   360

GRGKVGLSKLRYLVLDEADRMLDMGFEPDMRRLVGSPGMPSKENRQTLMFSATYPEDIQR   420
                  ATP-B
MAADFLKTDYLFLAVGVVGGACSDVEQTFVQVTKFSKREQLLDLLKTTGTERTMVFVETK   480

RQADFIATFLCQEKVPTTSIHGDREQREREQALADFRSGKCPVLVATSVAARGLDIPDVQ   540

HVVNFDLPSNIDEYVHRIGRTGRCGNTGRAVSFYDPEADGHLARSLVGVLSKAQQEVPSW   600

LEEAAFSGPSSTGFNPPRKNFASTDTRQRGLLQDTSVMSQPAAQPAADEEEWE*        660
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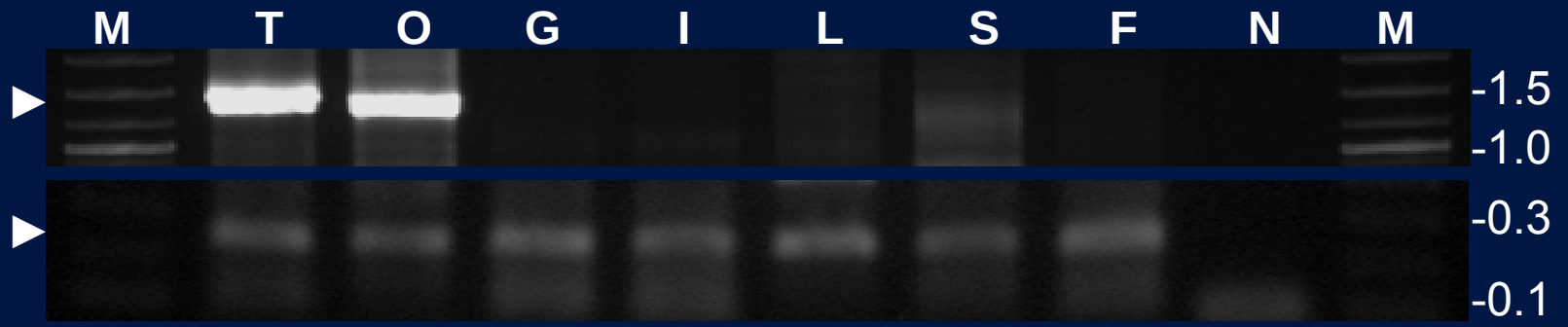
- Expression analysis: RT-PCR and *in situ* hybridization methods

RT-PCR analysis:

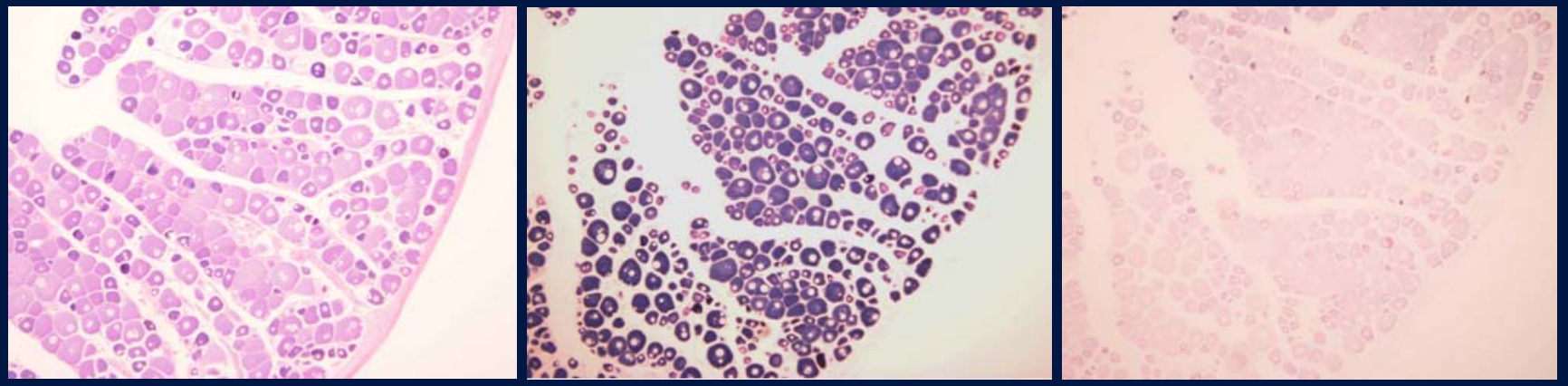


Ekspresi gen vasa pada testis (T), ovari (O), insang (G), usus (I), hati (L), otot (S), dan sirip (F) ikan gurame. M adalah marker DNA; N adalah produk PCR tanpa DNA cetakan.

In situ hybridization analysis:

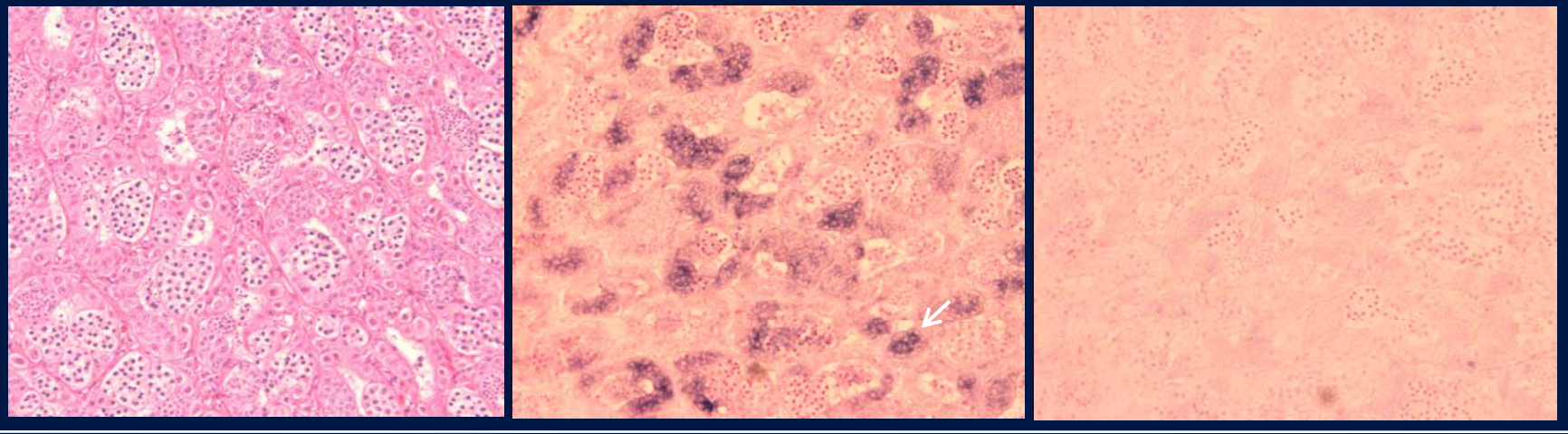
- Sintesis *in vitro* probe RNA : T7 polymerase
- Panjang probe RNA : 1,4 kb

H.E. antisense sense



ovary

H.E. antisense sense



testes

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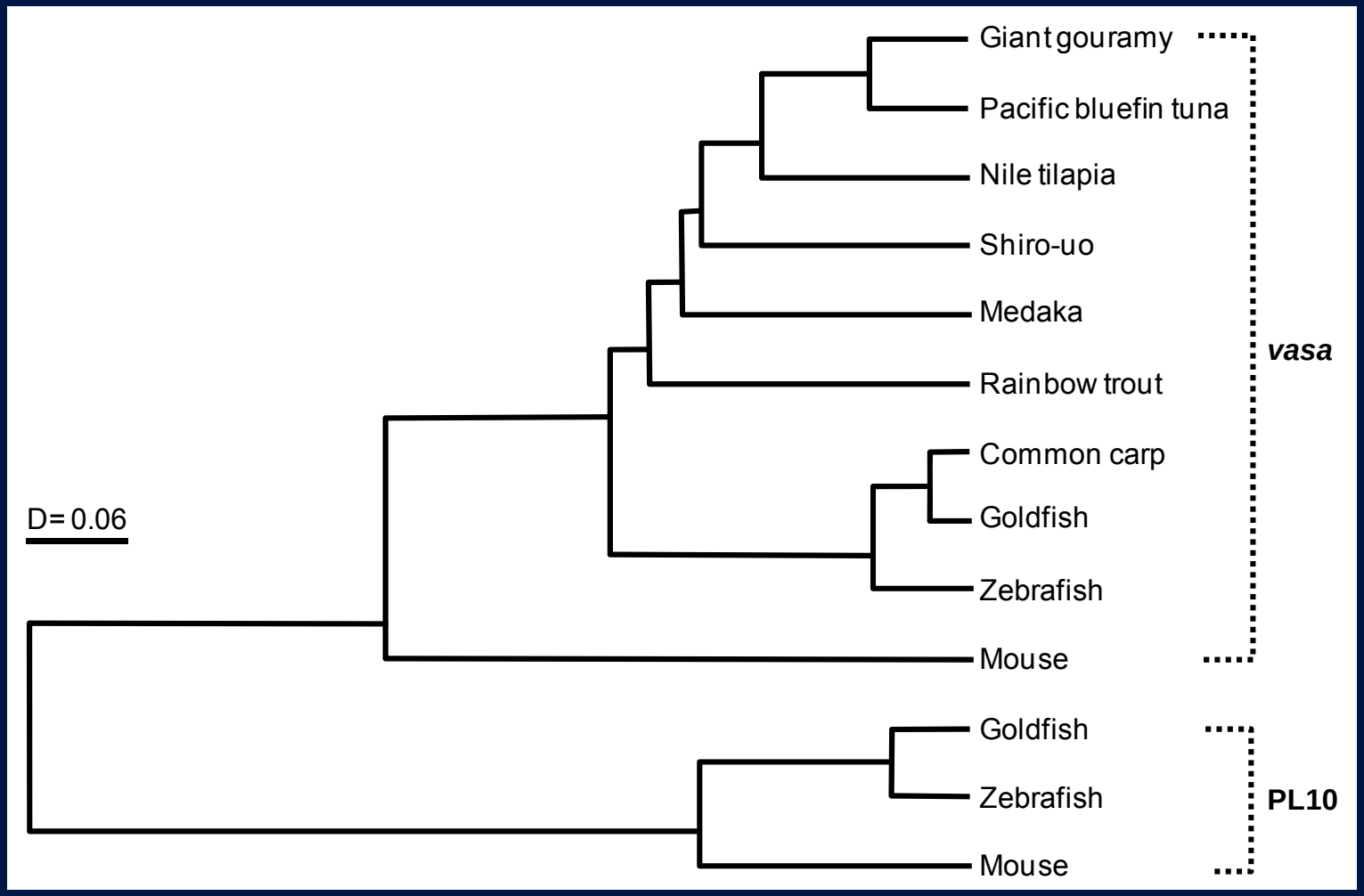
Phylogenetic tree:



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2. Development of DNA molecular marker in gonadal cell identification of giant gouramy (*Osphronemus gouramy*) and Nile tilapia (*Oreochromis niloticus*) using PCR

ABSTRACT

The technology of fish germ cell transplantation had been established to create broodstock systems by which a target offspring can be produced from a surrogate parent. This technique successfully applied in salmonid. Donor cell for transplantation is derived from transgenic fish carrying green fluorescent protein gene functions as a marker to distinguish the donor from recipient cell. In this study, we developed an alternative technique for identifying gouramy-derived donor cell and Nile tilapia as recipient by PCR amplification method using growth hormone (GH) and *vasa* genes as a molecular marker. β-actin gene was used as an internal control of DNA loading. The result showed that a specific PCR amplification product of 340 and 300 bp in length was obtained for GH and vasa, respectively. Both of evaluated molecular markers could be used to distinguish the donor cell, and GH marker showed higher sensitivity than vasa marker.

Keywords: transplantation, GH, vasa, marker, gouramy.

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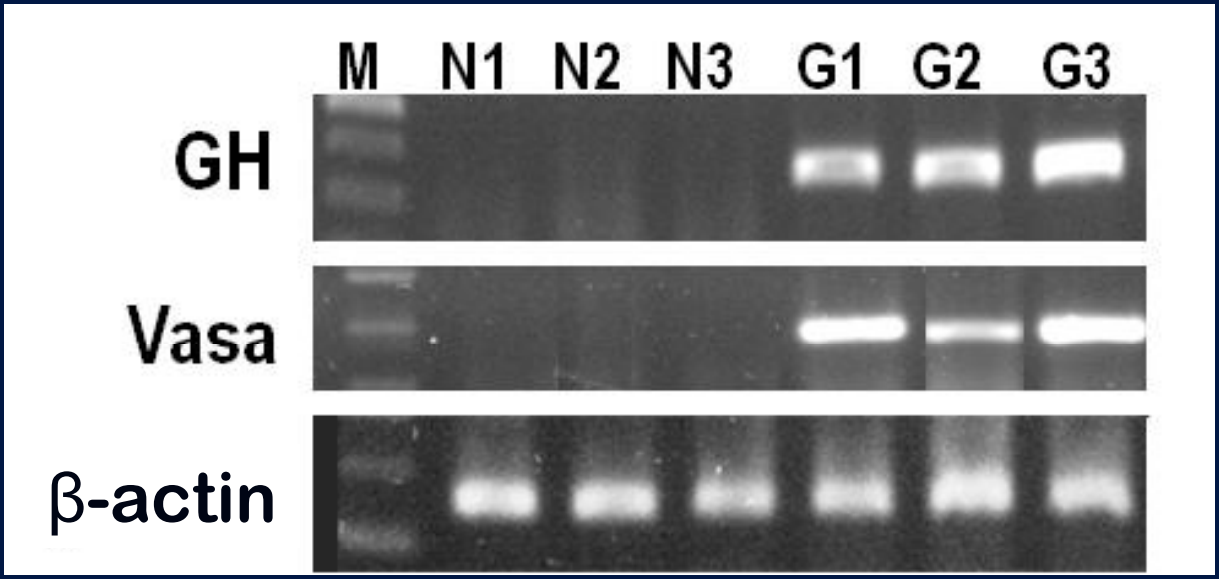
1. Specific primer

- Growth hormone gene, GH (Nugroho *et al*., 2008)
- *Vasa-like* gene (This study)

Software: GENETYX Ver. 7

G (5'-TGTTCTCTGACG-3')
N (5'-GCAACAAAAAA-3')

F1GH (5'-TGTTCTCTGACGGCGTGGTT-3')
R1GH (5'-GCAACAAAAAACCACCAGAAAGAG-3'),
F2VSGR (5'-TGAAGAAGAGTGGGAGTAGAAGG-3')
R3VSGR (5'-ACGTTCTGTCTGTCAGACACATTG-3);
β-aktin F (5'-GTGCCCATCTACGAGGGTTA-3'),
β-aktin R (5'-TTTGATGTCACGCACGATTT-3').

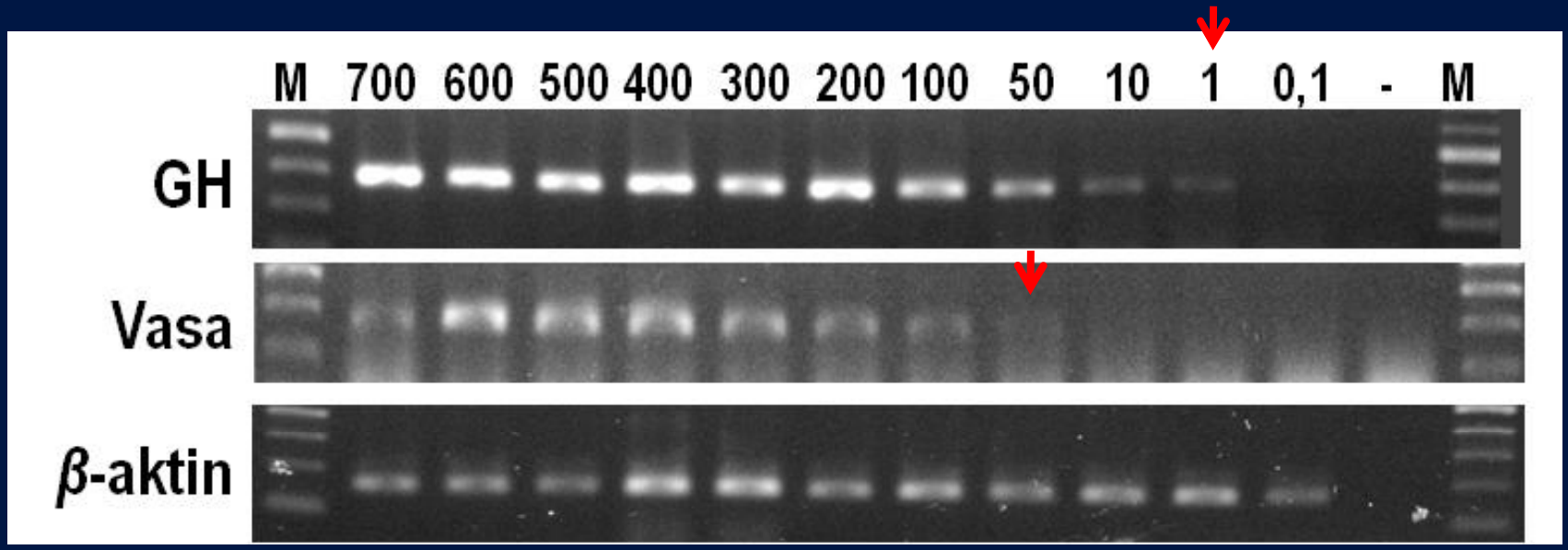


M: Marka ukuran fragmen DNA
N: DNA ikan nila
G: DNA ikan gurame

Gen GH dan gen *vasa* dapat digunakan sebagai marka molekular

2. PCR sensitivity:

0,1 – 700 ng/µl DNA genom ikan gurame dicampur dengan 700 ng/µl DNA genom ikan nila



- Primer dari gen GH lebih sensitif dibandingkan dengan gen *vasa*
- Konsentrasi DNA minimal dapat terdeteksi 1 ng/µl (GH), 50 ng/µl (*vasa*)

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Penelitian Selanjutnya:

1. Produksi ikan resipien untuk pengujian diferensiasi/proliferasi dan uji fungsional
2. Transplantasi menggunakan sel donor dari ikan gurame ukuran sekitar 500 g/ekor
3. Analisis kolonisasi sel donor dalam individu resipien menggunakan metode PCR
4. Analisis ekspresi *vasa* mRNA di PGC dengan metode hibridisasi *in situ* untuk publikasi

Acknowledgement:

1. DIPA IPB No.: 50/13.24.4/SPK/BG-PSN/2009
2. Irma Andriyani (S3 PS AKU, IPB)
3. Marlina Ahmad (S2 PS AKU IPB, sudah lulus)
4. Mauluddin (S1 BDP, sudah lulus)
5. Prof. Dr. Goro Yoshizaki (Tokyo University of Marine Science & Technology)
6. Balai Besar Pengembangan Budidaya Air Tawar Sukabumi , DKP

TERIMA KASIH