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Production of Offspring Derived from Frozen Whole Fish Kept in Freezer

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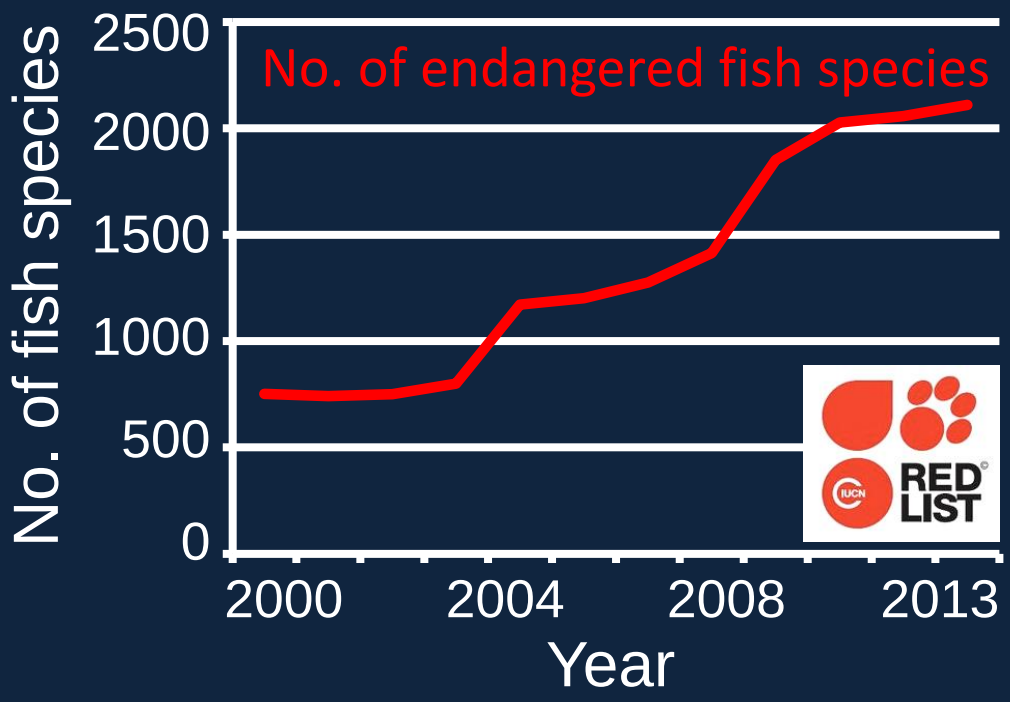
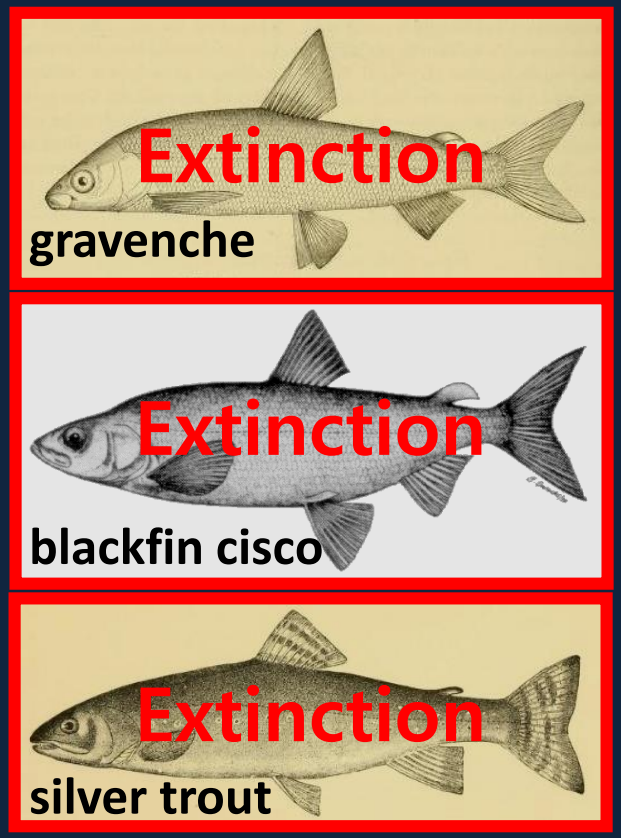
Global Fish Crisis



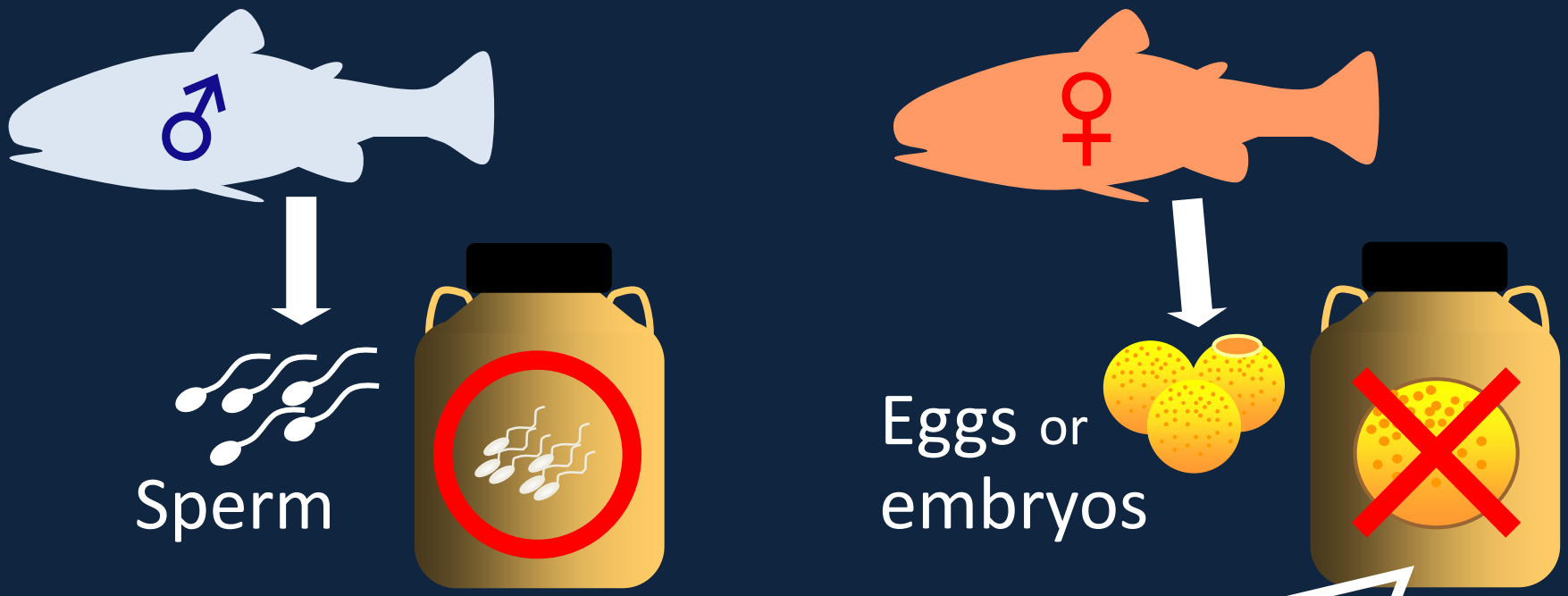


Growing numbers of fish species have become endangered.

Long-term preservation of fish genetic resources is essential.

Cryopreservation of fish gametes
would be a powerful tool to store
the fish genetic resources semi-permanently.

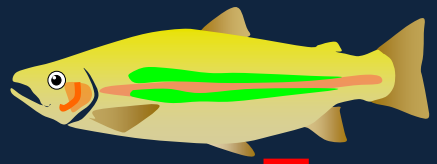
Preservation of fish genetic resources



Due to their large size and high yolk content

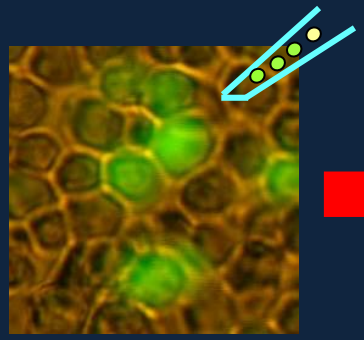
Maternally-inherited cytoplasmic compartments such as mitochondrial DNA can not be preserved !

Albino P*vasa-gfp*
donor trout

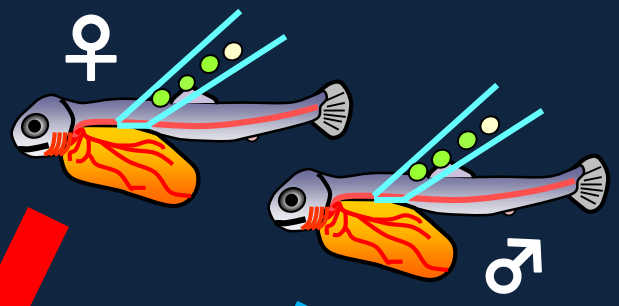


Testis

Testicular cells



Transplantation into
triploid recipients

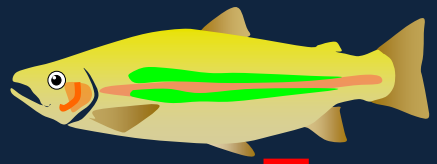


Production of only donor-derived gametes

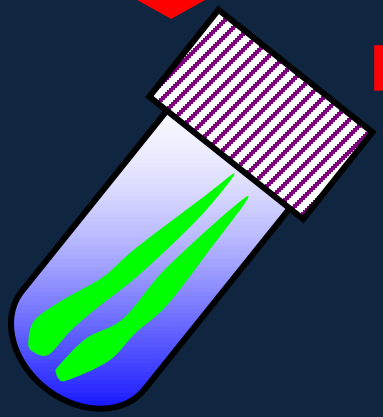
Okutsu et al. PNAS (2006)

Okutsu et al. Science (2007)

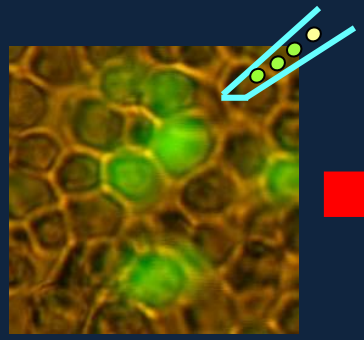
Albino P*vasa-gfp* donor trout



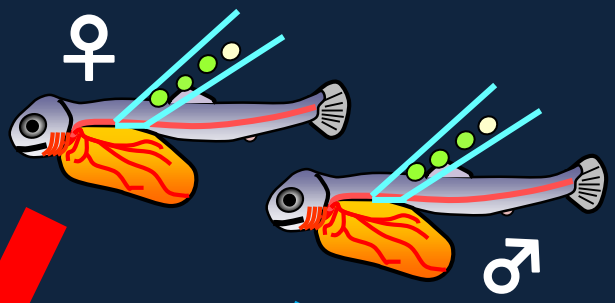
Whole testis cryopreservation



Testicular cells



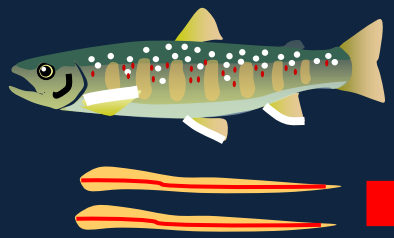
Transplantation into triploid recipients

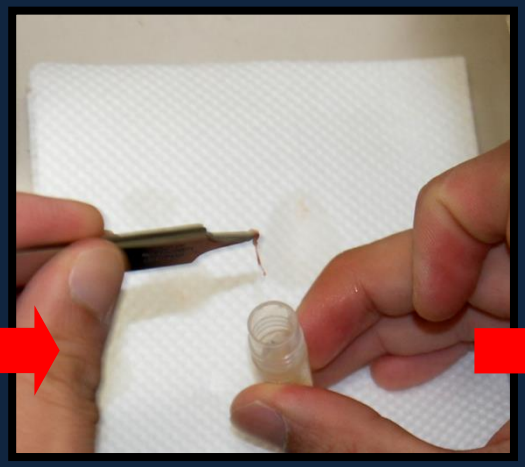


Generation of functional eggs and sperm from cryopreserved whole testes

Lee et al. PNAS (2013)

Endangered fish





Testes are transferred into cryotube containing cryomedium & equilibrated for 60 min

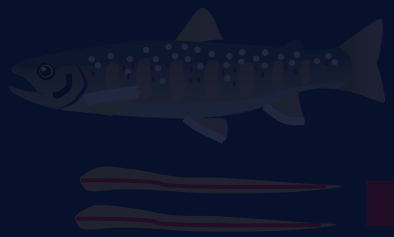
Cryotubes are transferred into slow-freezing container

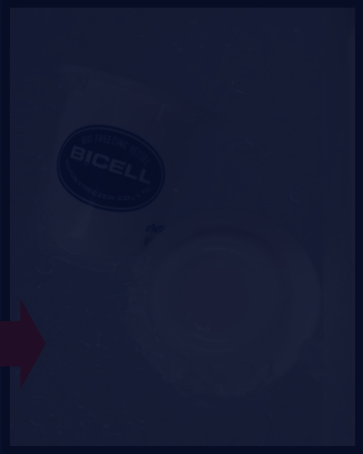
Freezing at a rate of -1°C/min in deep freezer for 90 min

Plunging into LN_2

Storage in LN_2 tank

Endangered fish

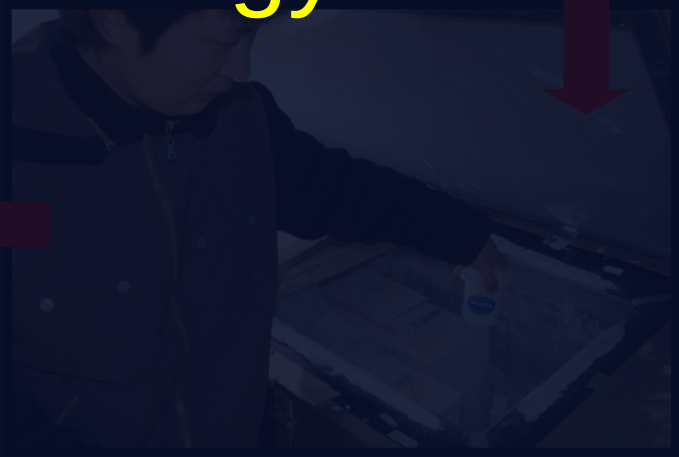




Testes are transferred into cryotube containing cryomedium & equilibrated for 60 min

Cryotubes are transferred into slow-freezing container

However, in order to expand the use of this technology



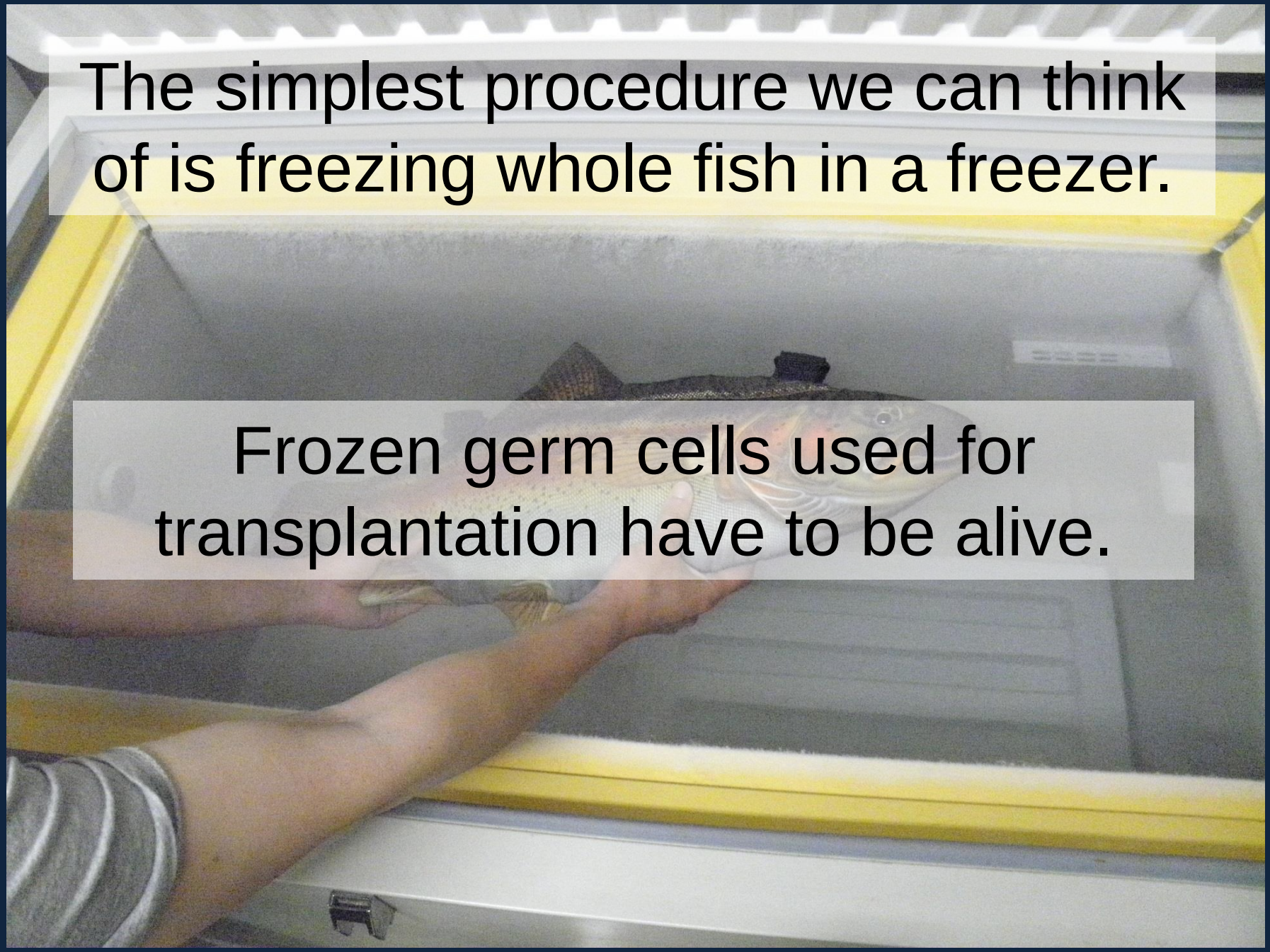
Storage in LN_2 tank

Plunging into LN_2

Freezing at a rate of -1°C/min in deep freezer for 90 min

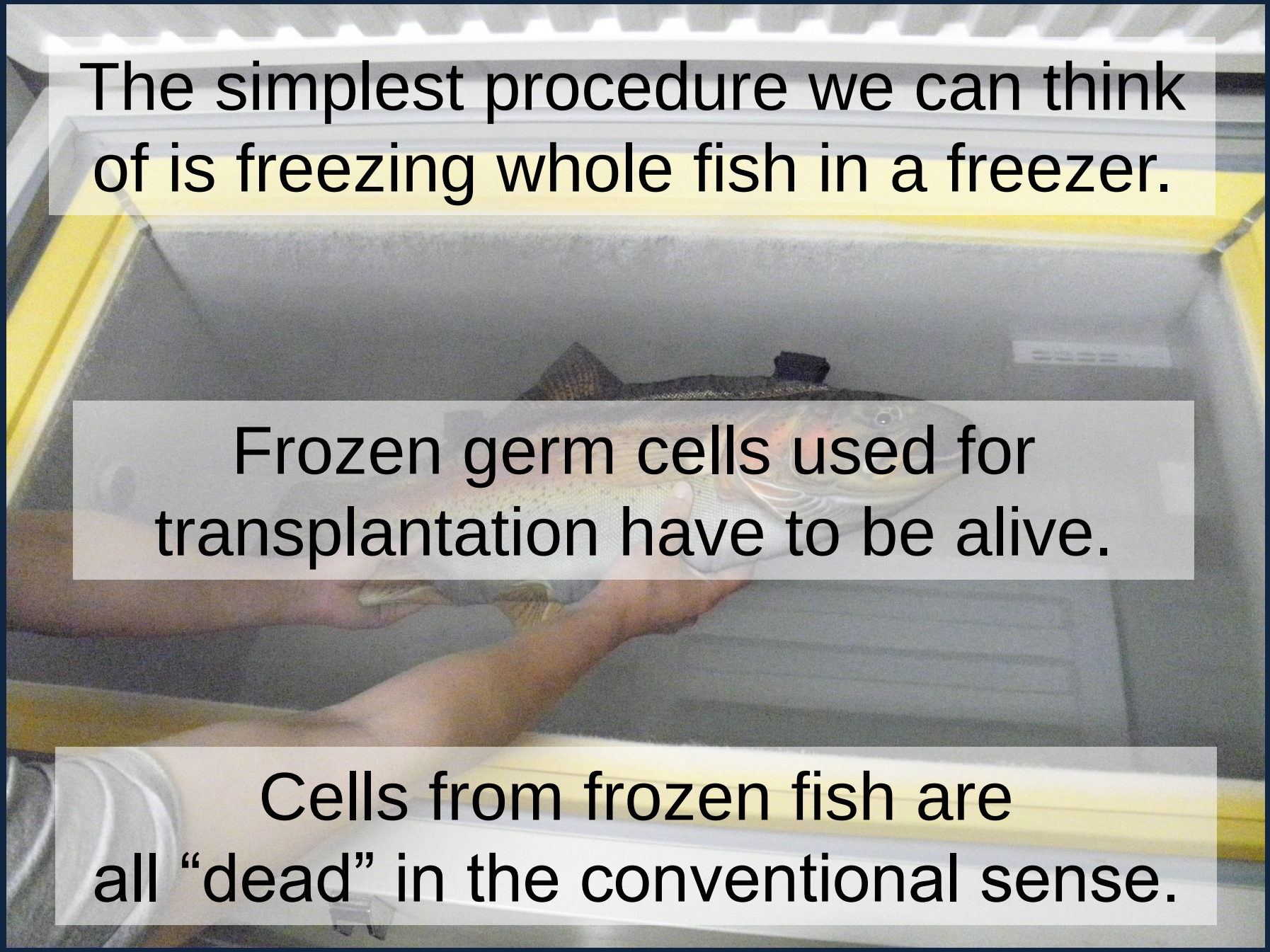
A simpler method is preferable.

The simplest procedure we can think of is freezing whole fish in a freezer.



The simplest procedure we can think of is freezing whole fish in a freezer.

Frozen germ cells used for transplantation have to be alive.



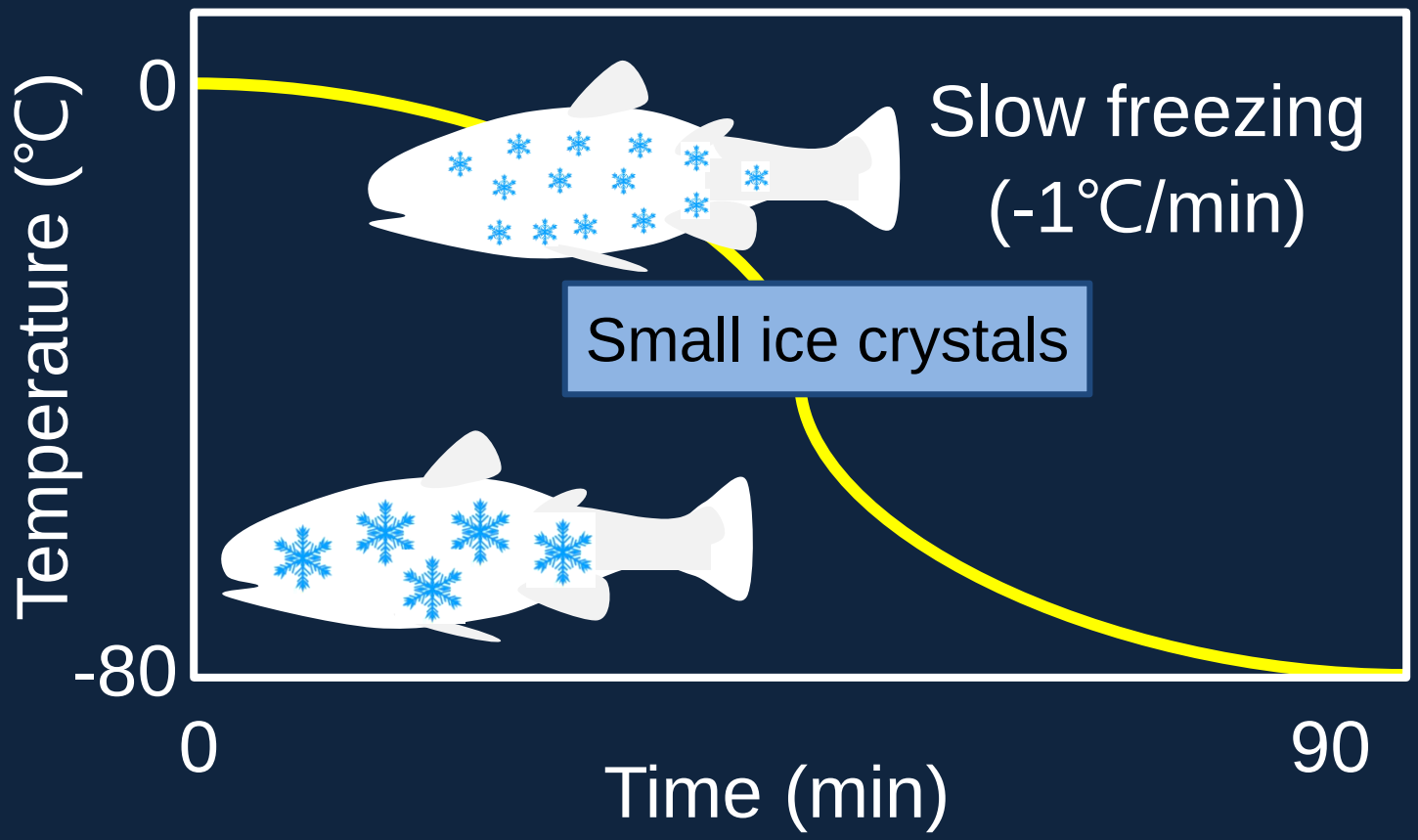
The simplest procedure we can think of is freezing whole fish in a freezer.

Frozen germ cells used for transplantation have to be alive.

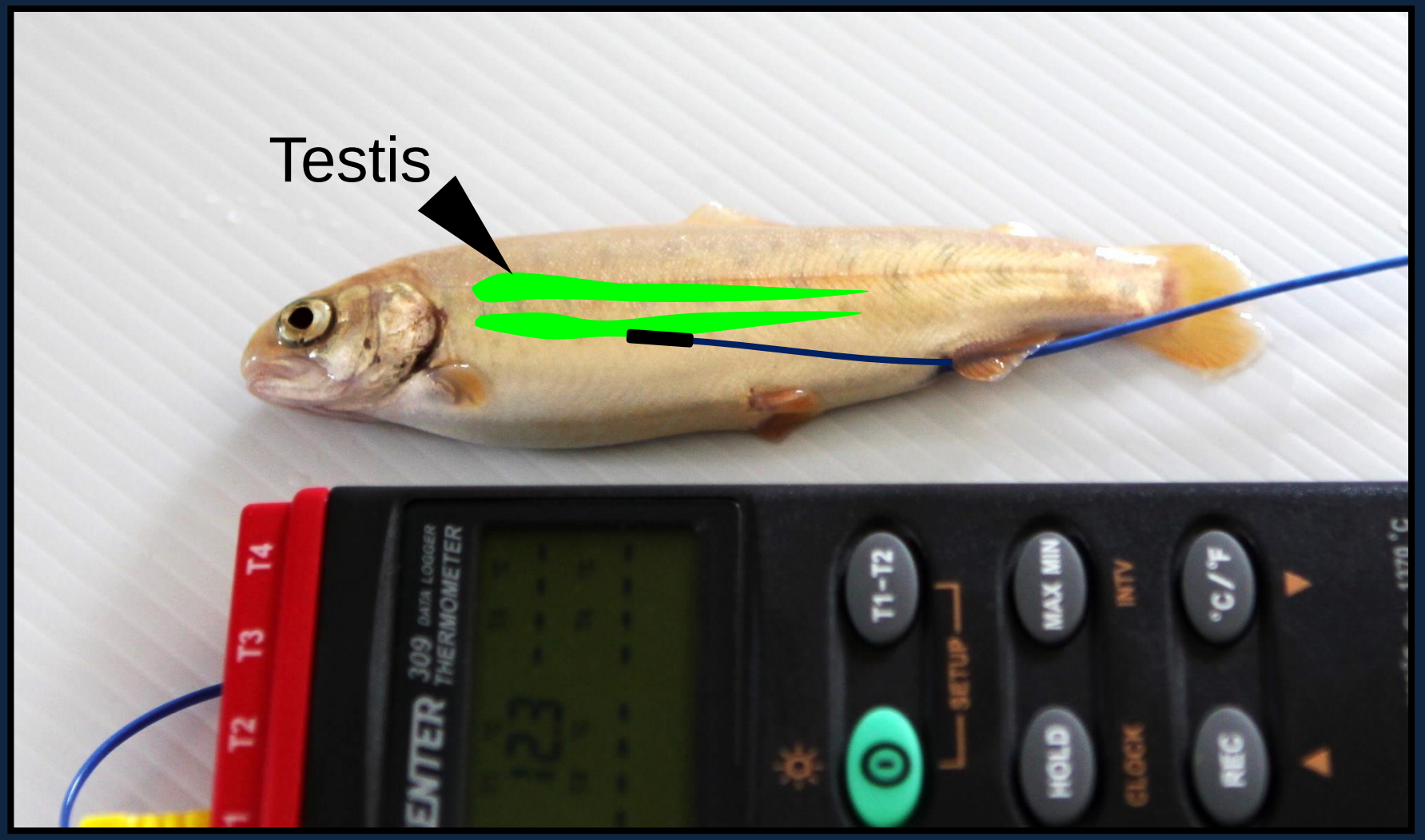
Cells from frozen fish are all “dead” in the conventional sense.

(Q1) Are germ cells retrieved from frozen whole fish alive?

Slow-freezing is required.



This is a widely used method
to obtain high viability after freezing.



Temperature changes inside of whole trout were measured during the freezing process.

Whole fish freezing

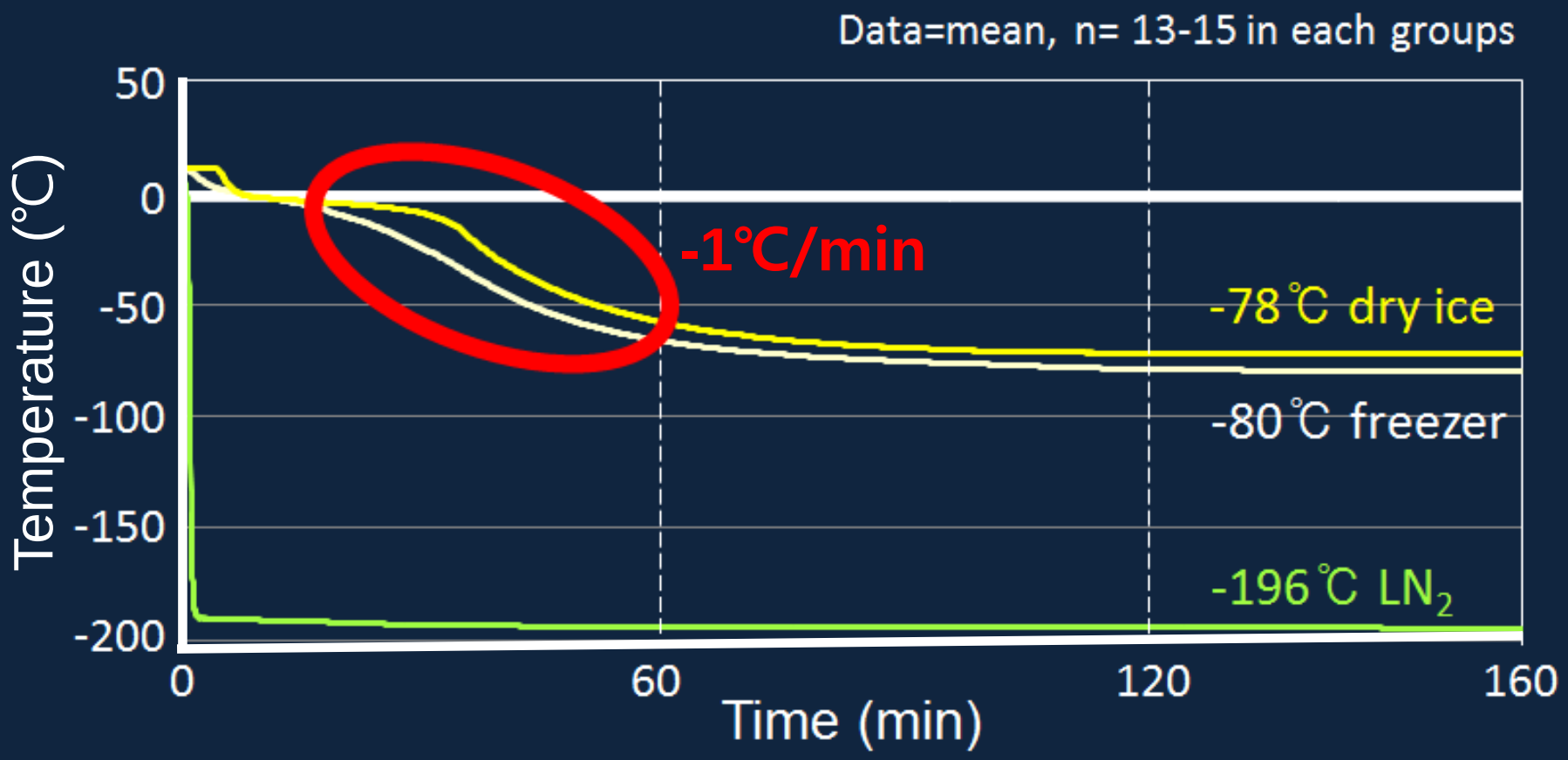
With thermometer

Albino
P*vasa-gfp* trout

BW: 24.5 ± 2.8 g
SL: 11.8 ± 0.9 cm
n= 13-15 in each groups

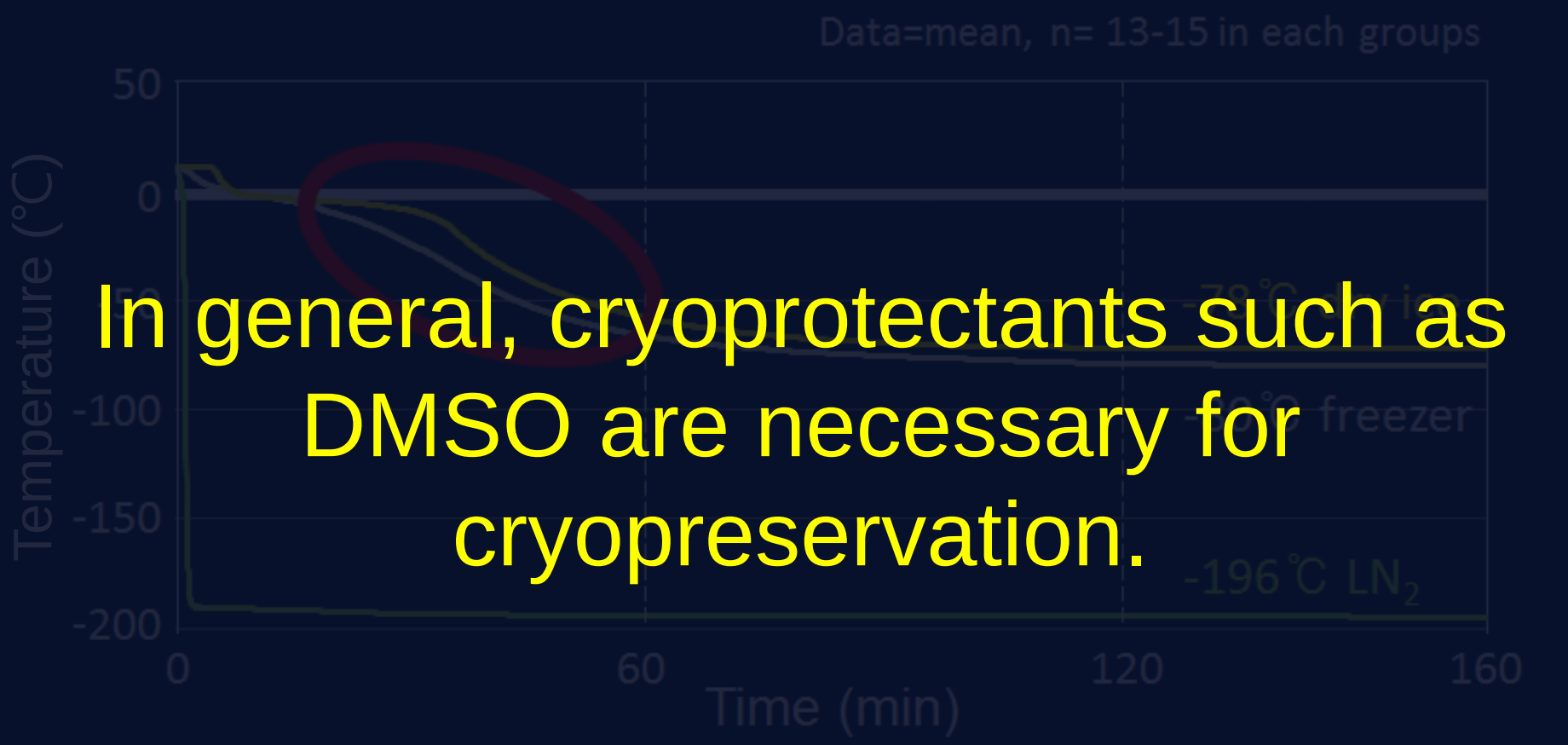
Whole fish freezing w/o any cryo-protectants

Temperature changes of SGs during whole fish freezing



Whole fish freezing can reproduce “-1℃/min slow-freezing” without using any cryo-container nor program freezer.

Temperature changes of SGs during whole fish freezing



In general, cryoprotectants such as DMSO are necessary for cryopreservation.

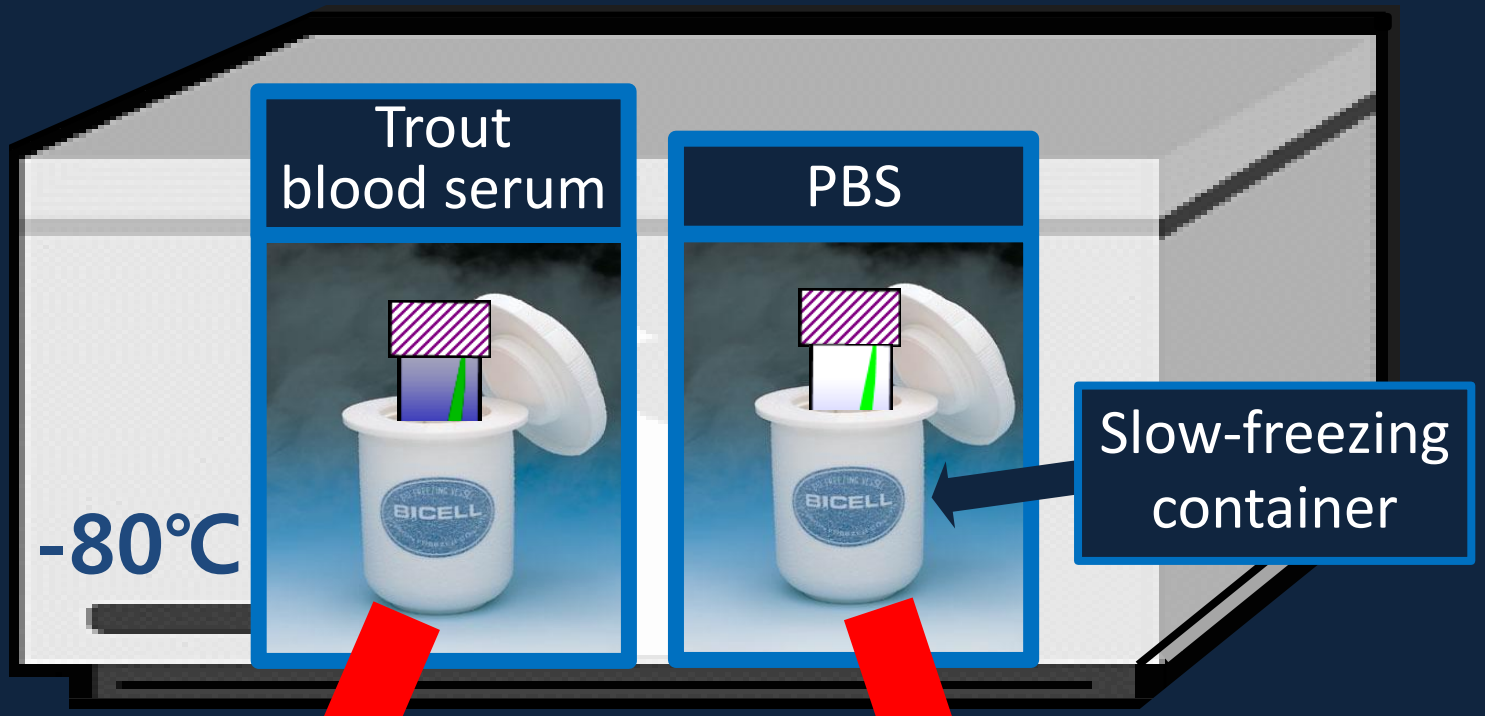
Whole fish freezing can reproduce “-1℃/min slow-freezing” without using any cryo-container nor program freezer.

(Q1) Are germ cells retrieved from frozen whole fish alive?

Slow-freezing can be reproduced in fish body.

(Q2) Can we cryopreserve germ cells without cryo-protectants?

Slow-Freezing of testis for 1 day



Data=mean±SEM, n= 11

Trout blood serum

592±127 SGs/fish

PBS

14±5 SGs/fish

Trout blood serum can be worked as a cryo-protectant.

(Q1) Are germ cells retrieved from frozen whole fish alive?

Slow-freezing can be reproduced in fish body.

(Q2) Can we cryopreserve germ cells without cryo-protectants?

Blood serum can be worked as a cryo-protectant.

(Q1) Are germ cells retrieved from frozen whole fish alive?

(Q2) Can we cryopreserve germ cells without cryo-protectants?

(Q3) Can we retrieve live germ cells from frozen whole bodies of rainbow trout?

Whole fish freezing

Albino
P*vasa-gfp* trout

BW: 26.2 ± 3.9 g
SL: 12.6 ± 1.0 cm
n= 18-25 in each groups

Whole fish freezing w/o any cryo-protectants for 1 W

Whole fish thawing

Thawed by immersing in a 10℃ water bath for 5 min

Testis isolation and dissociation

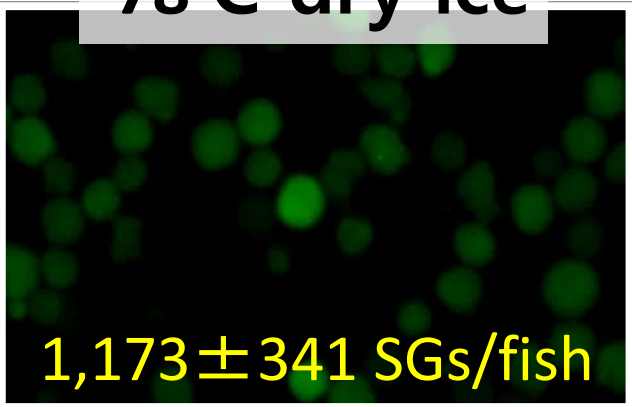
Viability of SGs following freezing conditions

Albino
P*vasa-gfp* trout

BW: 26.2 ± 3.9 g
SL: 12.6 ± 1.0 cm
n= 18-25 in each groups

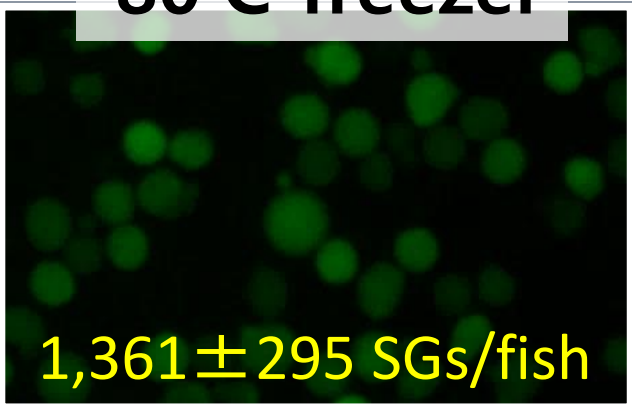
Whole fish freezing w/o any cryo-protectants for 1 W

-78℃ dry ice



1,173±341 SGs/fish

-80℃ freezer



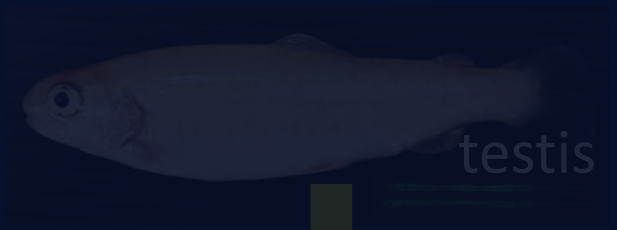
1,361±295 SGs/fish

-196℃ LN_2

0 SGs/fish

Viability of SGs following freezing conditions

Albino
P*vasa-gfp* trout



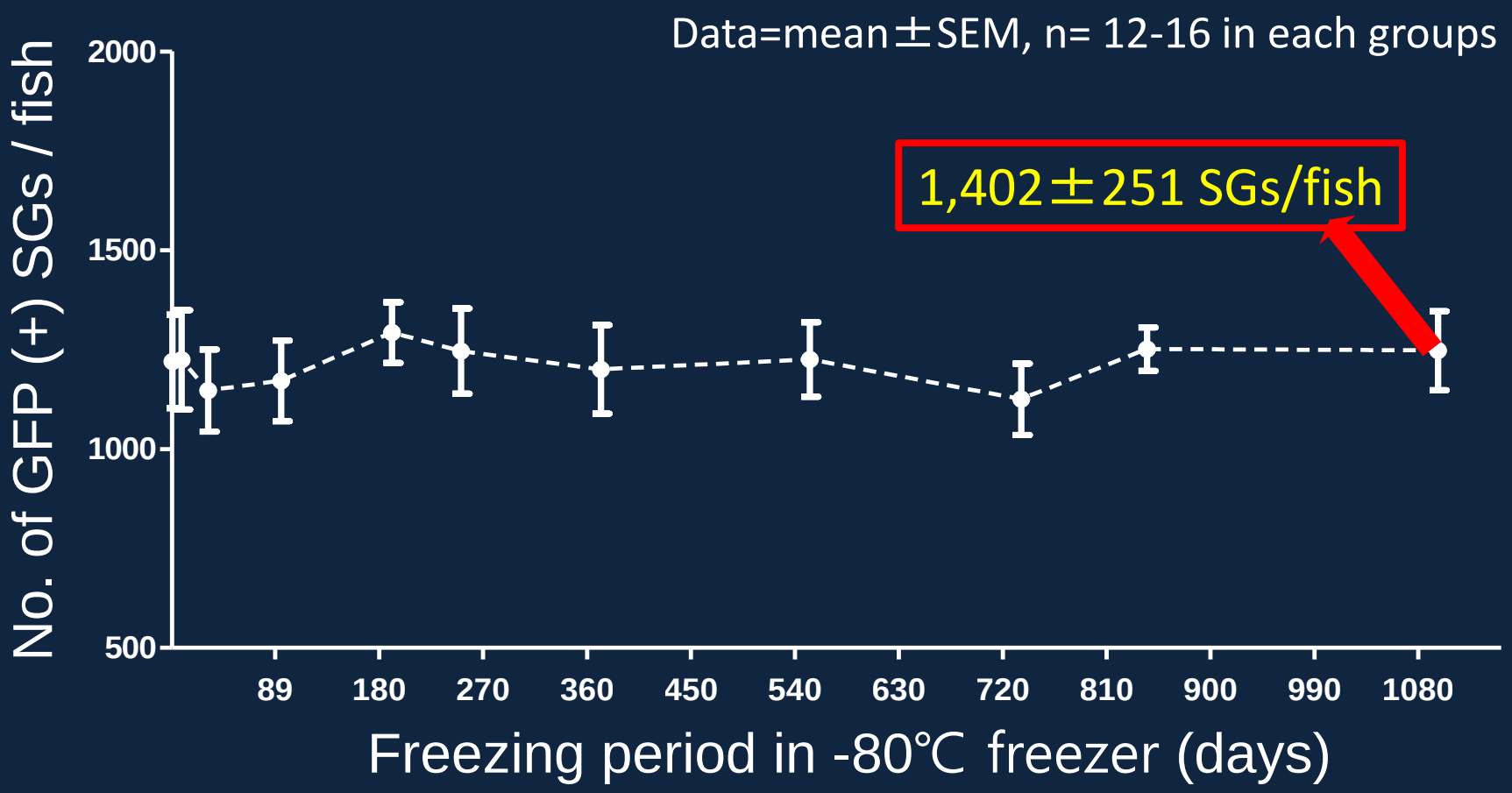
BW: 26.2 ± 3.9 g
SL: 12.6 ± 1.0 cm
n= 18-25 in each groups

Whole fish freezing w/o any cryo-protectants for 1 W

Can spermatogonia inside of frozen whole fish be preserved for a long time?

-78℃ dry ice	-80℃ freezer	-196℃ LN_2
1,173±341 SGs/fish	1,361±295 SGs/fish	0 SGs/fish

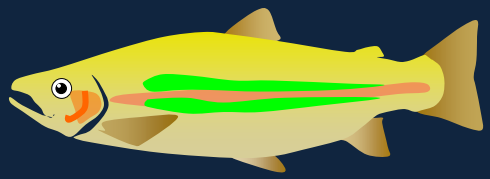
Viability of SGs following freezing periods



Viability of SGs obtained from frozen whole fish did not vary with changes to the duration of freezing.

Transplantation of frozen-thawed spermatogonia

Whole fish freezing for 3 y

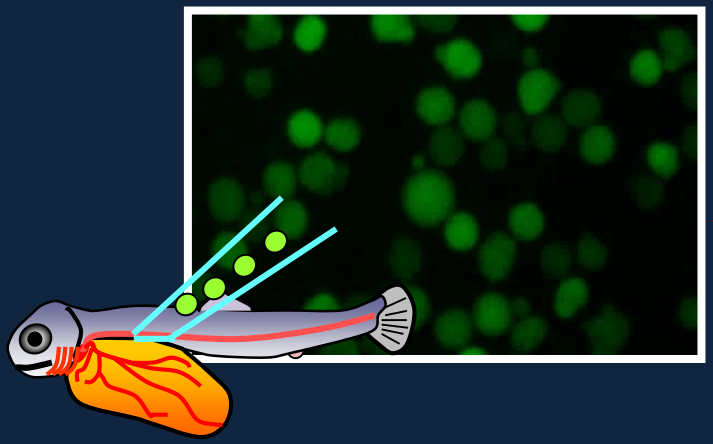


Heterozygous albino hemizygous P*vasa-gfp* donor rainbow trout

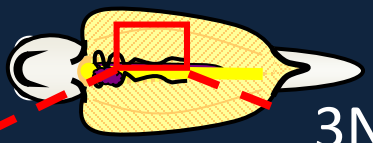
Thawing in a 10℃ water bath

Testis isolation from thawed fish

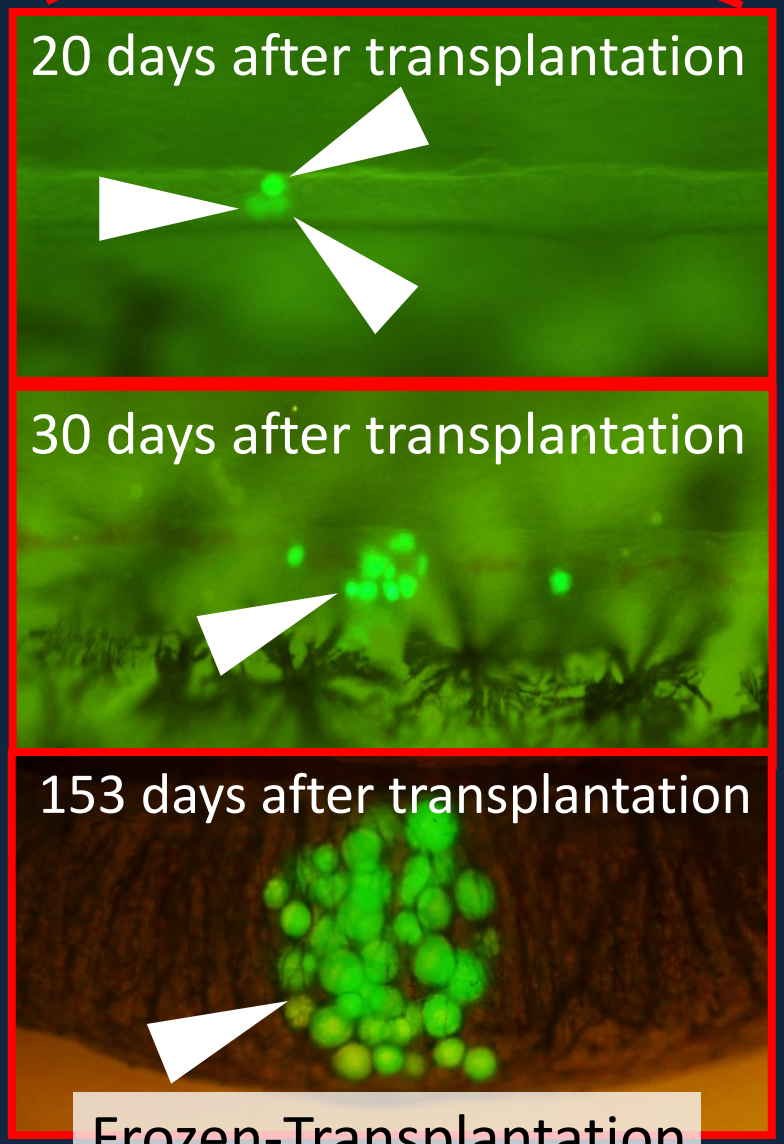
Dissociation

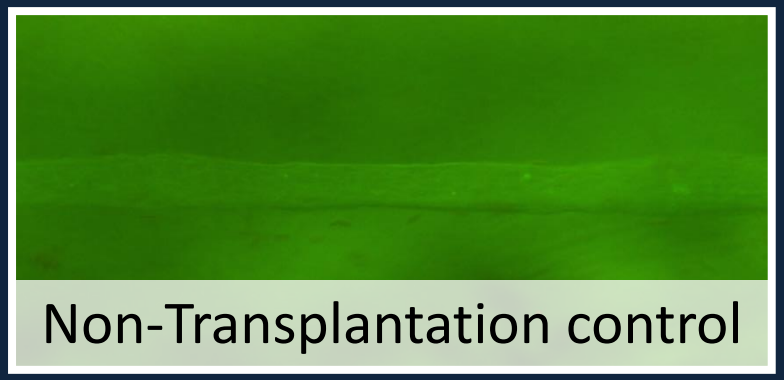


Transplantation of 500 SGs into WT 3N masu salmon



Wild-type
3N salmon recipient

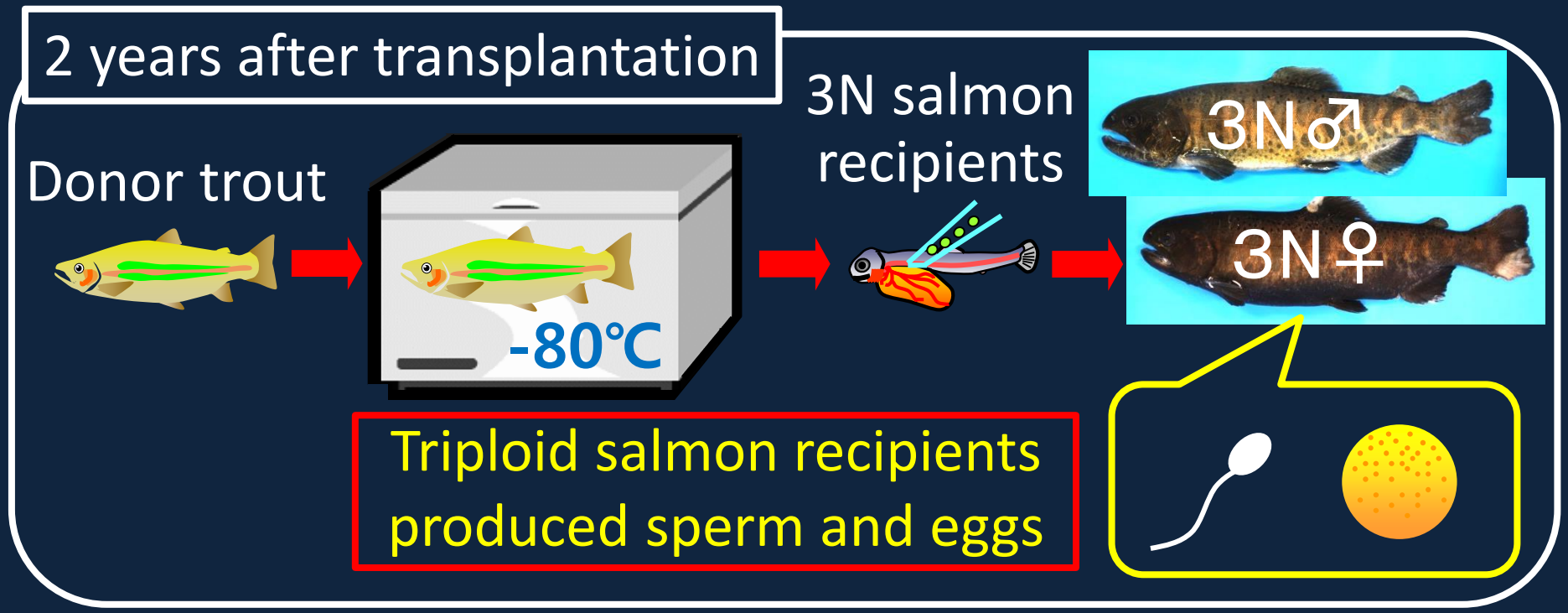




Group	No. (%) of recipients with trout GFP (+) SGs
Frozen	74/130 (56.9±6.7%)
Fresh	81/129 (62.8±4.1%)
Non-TP	0/30

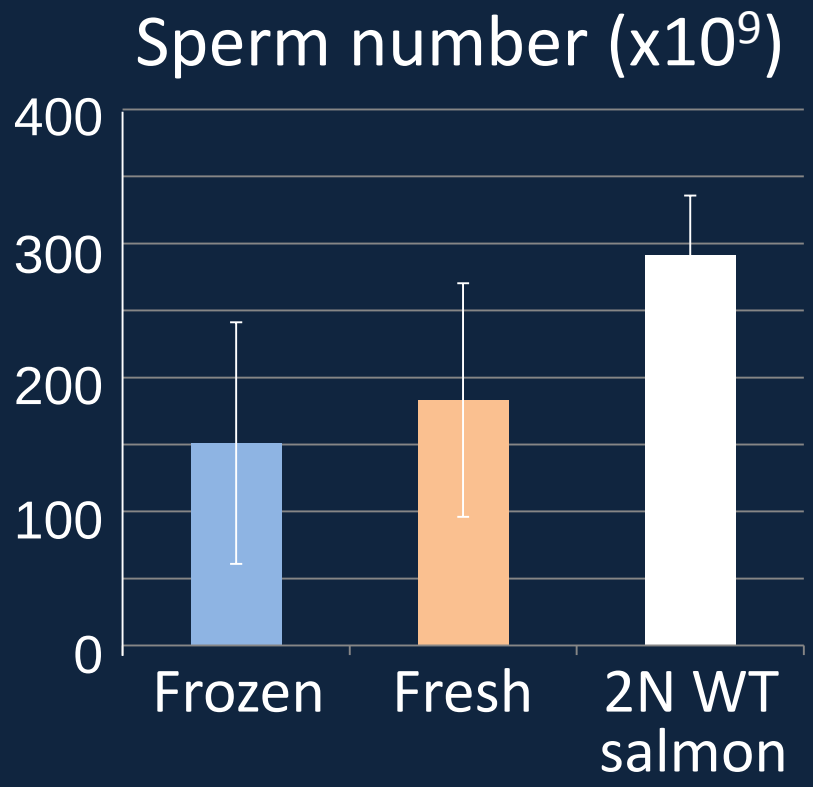
Data=mean±SEM, $P < 0.05$

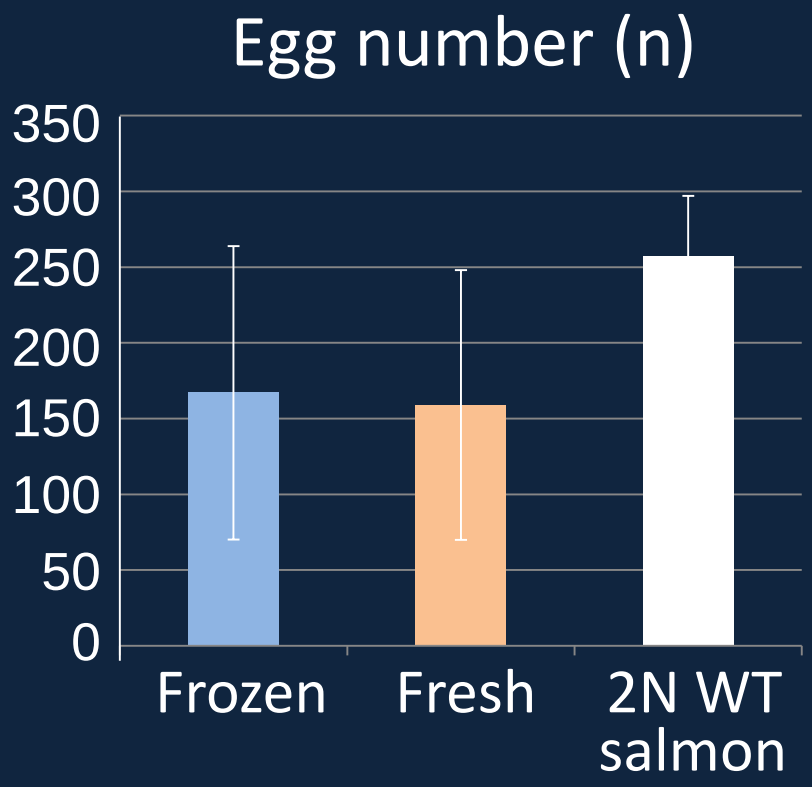
Maturation of triploid salmon recipients



Group	Mature fish (%)	
	Male	Female
Frozen	4/13 (30.8%)	2/12 (16.7%)
Fresh	4/12 (33.3%)	3/17 (17.6%)

No. of gametes obtained from salmon recipients

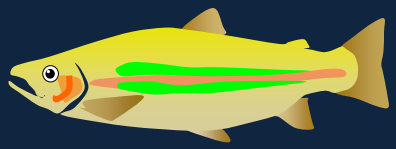


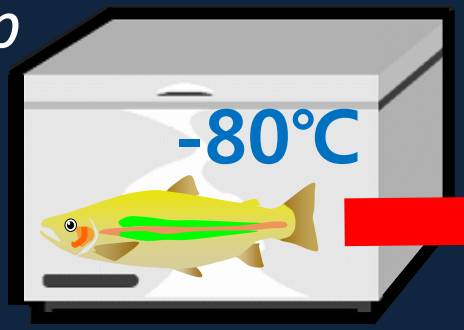


Data=mean±SD, $P < 0.05$

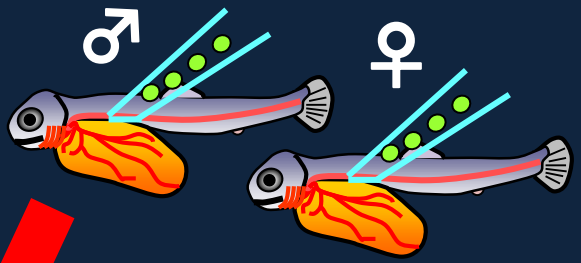
Sperm and egg numbers obtained from frozen transplants were not significantly different from other groups.

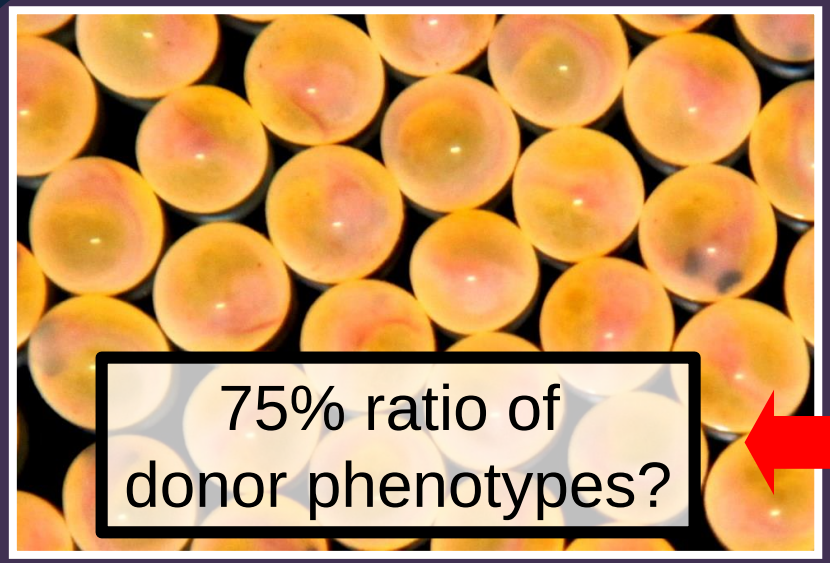
Heterozygous albino hemizygous P*vasa-gfp* donor rainbow trout





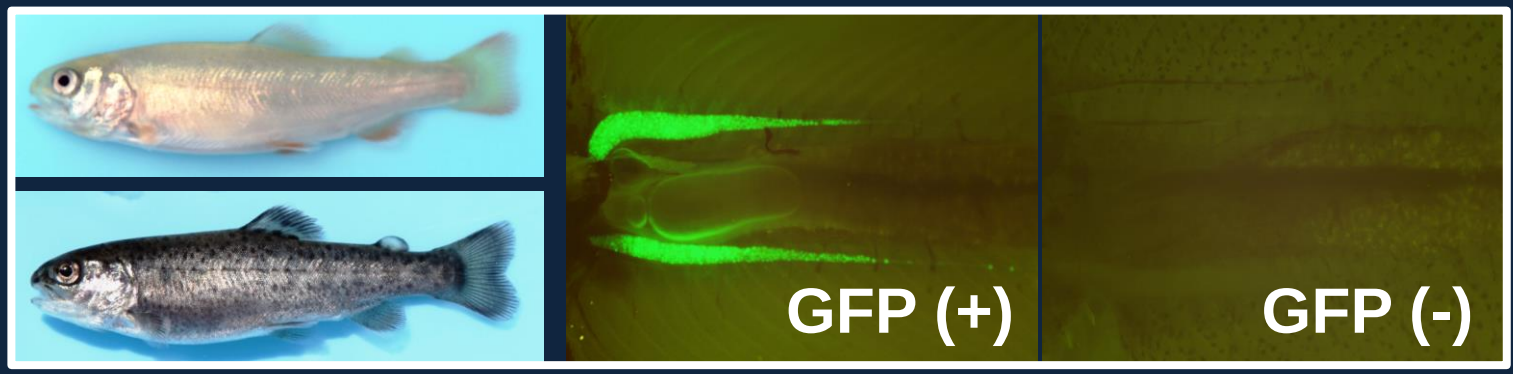
Transplantation into WT 3N masu salmon





Can gametes obtained from triploid recipients generate offspring derived from frozen whole trout?

Phenotypic analysis of F1 offspring

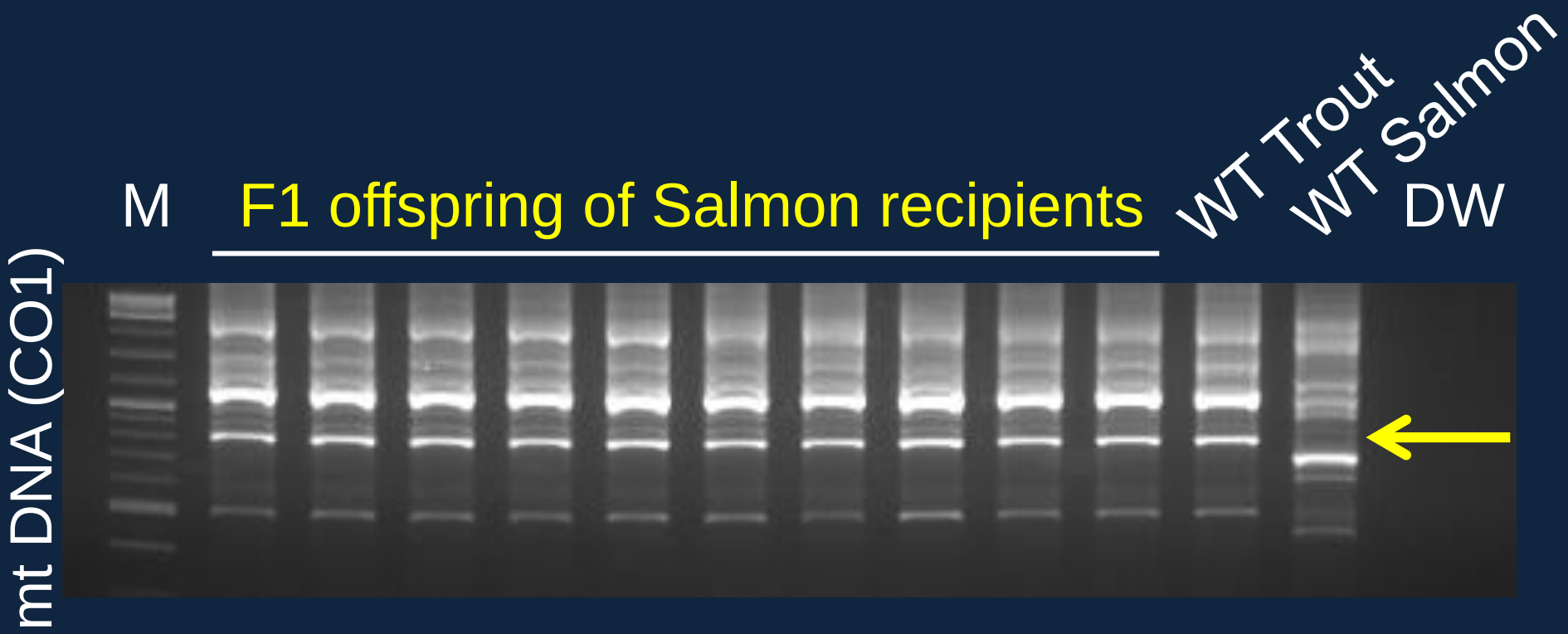


Karyotypic analysis of F1 offspring

2n=60
(WT trout)

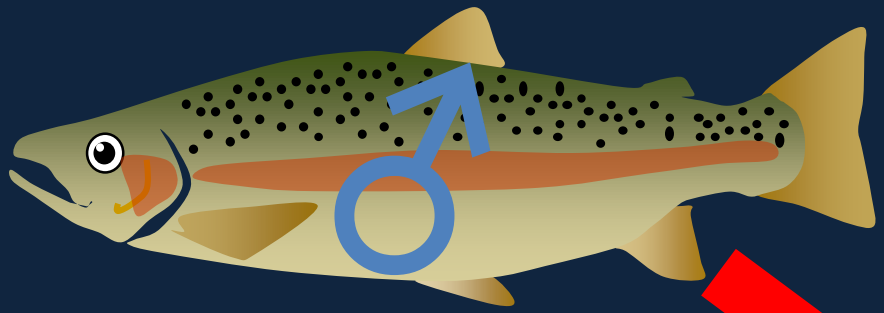
2n=66
(WT salmon)

RAPD analysis of F1 offspring



F1 offspring were successfully derived
from frozen whole fish by interspecies transplantation !

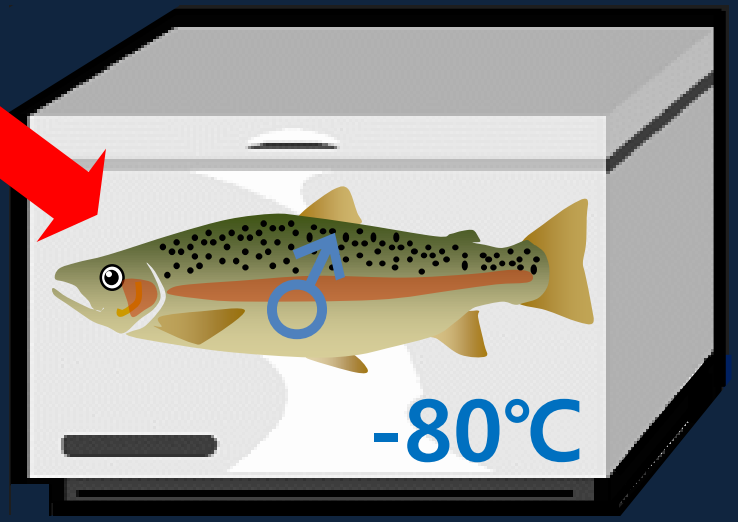
Conclusion



w/o cryo-protectants
w/o slow-freezing container
w/o LN_2

Freezing efficiency
Isolated testis > whole fish freezing

Whole fish freezing is a convenient emergency tool.



Applicable to regenerate extinct fish species when they are stored in a deep freezer.


Thank you for your attention.