The function of wax esters in larval fish transition from endogenous to exogenous nutrition: fish, exception or the rule?

Konrad Dabrowski

Ghent University, Belgium, 2-5 September 2013
The function of wax esters in larval fish transition from endogenous to exogenous nutrition. Are freshwater fish the exception or the rule?

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Department of Evolution, Ecology and Organismal Biology, OSU
Plan of presentation

1. Oogenesis and early life history of yellow perch
2. Yolk utilization and critical points in early life history
3. Function of lipids and fatty acids in larval fish viability
4. Materials and Methods
5. Results
6. Lipids classes and fatty acid in yellow perch
7. Why waxes?
8. New research directions to link perch waxes to physical environment during early life and recruitment
Yellow perch oogenesis in North America
(Malservisi and Magnin 1968. Nat. Canad.)

Lipid droplets (gl), yolk Vesicles (v), nucleus (N).

Oocyte in the process of hydration,
Oil globule (GL), pervitelline space (EPV), germonal cell (PG).
Perch (*Perca flavescens*) blastula........ 100% fertilization
Oil globule utilization in striped bass larvae
(Eldridge et al. 1981. TAFS 110:111)

Larva starving for 12 days after hatching

Larva feeding for 12 days after hatching
Swim bladder inflation process and physical conditions accompanying the successful event in walleye (Regier and Summerfelt 2001. J.Fish Biol. 53:93)

1. Water surface tension
2. Duration of pneumatic duct opening
3. Swim bladder lipid content and profiles
Are there alternative strategies for larvae of different fish species that do not rely on maternal phospholipids?


“Marine fish phospholipids: the gold standard in larval fish nutrition”

- Fish larvae have a limited ability to biosynthesise phospholipids de novo but can exchange fatty acids within and between PL and TG.
- Dietary phospholipids can, in principle, be utilized (assimilated) unchanged.
- Ideal larval diet is the one that matches yolk sac and natural exogenous diet lipid composition.

**Effect of snook eggs DHA level on hatching rate in 2002 - 2005**

Conclusions:
1. Low ARA and DHA reduced escape behavior in larvae
2. Reduced maternal essential FA was not compensated in initial feeding

Conclusions:
1. Polyunsaturated fatty acid (PUFA) are at higher concentration in PL than in NL.
2. Lipid content was unrelated to egg size.
3. PUFA concentration play no role in high viability of eggs (90%).
The role of wax esters in marine food chains, phytoplankton, copepods, fish (Benson 1975. Scientific American)

*Calanus plumchrus*
Wax esters biological insights and advantages over triglycerides and phospholipids!

1. Hydrophobic lipids in the larval fish (swim bladder and pneumatic duct) will facilitate inflation by acting as surfactants (lubricants) and preventing inner surfaces adhering and collapsing.

2. Osmoregulatory functions of larval fish enhanced (preventing transepidermal water rush)

3. Preventive role in bacterial and viral attachments and infections

4. Waxes have lower susceptibility to oxidation than other lipids (higher stability in low temperatures).

Hills 2002
Comparative aspects of lipid mobilization, transport and utilization (digestion) – does “arowana” model applies to wax esters in “oil globule” possessing larval fish?

Mobilization of wax esters contained in yolk oil globule may proceed through earlier described “endocytotis” of microparticles (decreased polarity compared to TG) into circulation....

The fate of dietary lipids, synthesis, transfer, accumulation and metabolism in fish (Wiegand 1996. Reviews in Fisheries)
Pathways for the biosynthesis of C20 and C22 HUFA

Linoleic acid
18:2n-6
\[ \Delta 6 \]
18:3 \[ \rightarrow \]
20:3 \[ \rightarrow \]
18:4 \[ \rightarrow \]
20:4

Linolenic acid
18:3n-3
\[ \Delta 6 \]
18:4 \[ \rightarrow \]
20:4

20:4n-6 Arachidonic AA
20:5n-3 Eicosapentaenoic EPA
22:6n-3 Docosahexaenoic DHA

\[ \Delta \] : microsomal fatty acil desaturase
CS : peroxisomal chain shortening
E : microsomal fatty acil elongase
Materials and Methods
## Experimental Design

<table>
<thead>
<tr>
<th>Fish origin</th>
<th>Winter Duration</th>
<th>Condition Entering Winter</th>
<th>Replication (N=3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Erie Domestic</td>
<td>50 days @ 4°C</td>
<td>Good</td>
<td>Each tank: 8 females, 4 males</td>
</tr>
<tr>
<td>Erie Domestic</td>
<td>80 days @ 4°C</td>
<td>Poor</td>
<td></td>
</tr>
<tr>
<td>Erie Domestic</td>
<td>110 days @ 4°C</td>
<td>Good</td>
<td></td>
</tr>
<tr>
<td>Erie Domestic</td>
<td>80 days @ 4°C</td>
<td>Poor</td>
<td></td>
</tr>
<tr>
<td>Erie Domestic</td>
<td>110 days @ 4°C</td>
<td>Poor</td>
<td></td>
</tr>
</tbody>
</table>

- Each tank: 8 females, 4 males
Laboratory Experiments

- Fall 2011 – Winter duration treatments began
Methods of analysis:
Lipid classes
Fatty acid composition

1. Separation of lipids into phospholipids (PL) and neutral lipids (NL)
2. Separation of the neutral lipid classes, triglycerides (TG) and wax esters (WE)
3. Methylation of fatty acids (FAME) and fatty alcohols (FAL).
Lipid extraction and separation

1. Homogenization

2. Clean-up

3. Separation

MgCl₂·6H₂O

Ice

Chloroform

Methanol

BHT

NL

PL
Lipid classes extraction and separation

1. Folch et al. (1957)

- Solvent 1. Hexane + 1% diethyl ether (Wax1+2)
- Solvent 2. Dichloromethane: hexane (1:2) (Wax1)
- Solvent 3. Dichloromethane: methanol (1:20) (PL)

Clean-up

Re-suspended in hexane

Chloroform

Methanol

NL

PL

Suspended in hexane

Methylation

Solvent 1. Dichloromethane (Fatty acids)
Solvent 2. Dichloromethane: ethyl ether (9:1) (Fatty alcohols)

Stevens, Parrish et al. 2004
Saito et al. 2000
Lipid classes and fatty acid composition of perch food (fathead minnows) offered in the course of gametogenesis 2011-2012

<table>
<thead>
<tr>
<th>Category</th>
<th>Mean %</th>
<th>Oct. 13*</th>
<th>Nov. 11*</th>
<th>Jan. 20†</th>
<th>Feb. 2‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Lipids</td>
<td></td>
<td>4.1</td>
<td>3.8</td>
<td>2.7</td>
<td>3.4</td>
</tr>
<tr>
<td>Neutral Lipids</td>
<td></td>
<td>69</td>
<td>67</td>
<td>58</td>
<td>64</td>
</tr>
<tr>
<td>Phospholipids</td>
<td></td>
<td>31</td>
<td>33</td>
<td>42</td>
<td>36</td>
</tr>
<tr>
<td>Linoleic C18:2 (n6)</td>
<td></td>
<td>0.05</td>
<td>0.02</td>
<td>0.02</td>
<td>0.07</td>
</tr>
<tr>
<td>Linolenic C18:3 (n3)</td>
<td></td>
<td>0.3</td>
<td>0.5</td>
<td>0.3</td>
<td>0.6</td>
</tr>
<tr>
<td>Arachidonic C20:5 (n3)</td>
<td></td>
<td>7.7</td>
<td>7.6</td>
<td>2.4</td>
<td>3.7</td>
</tr>
<tr>
<td>EPA C20:4 (n6)</td>
<td></td>
<td>0.01</td>
<td>0.03</td>
<td>0.03</td>
<td>0.02</td>
</tr>
<tr>
<td>DHA C22:6 (n3)</td>
<td></td>
<td>7.3</td>
<td>9.6</td>
<td>2.2</td>
<td>3.1</td>
</tr>
<tr>
<td>n3 / n6</td>
<td></td>
<td>9.4</td>
<td>13.2</td>
<td>6.5</td>
<td>4.3</td>
</tr>
</tbody>
</table>

* St. Mary’s State Fish Hatchery (ODOW)
† Jones Fish Hatchery (Cincinnati, OH)
‡ R&R Sports Headquarters (Columbus, OH)
Proportion of neutral lipids in oocytes (eggs) of fish (% total lipids)
(after Wiegand 1996 unless reference given)

<table>
<thead>
<tr>
<th>Lipid fraction</th>
<th>PL</th>
<th>NL</th>
<th>TG</th>
<th>WE</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Eggs with no oil globule</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cod</td>
<td>71.7</td>
<td></td>
<td>12.5</td>
<td>3.7</td>
</tr>
<tr>
<td>Atlantic halibut</td>
<td>70.8</td>
<td></td>
<td>12.9</td>
<td>4.3</td>
</tr>
<tr>
<td>Plaice</td>
<td>65.8</td>
<td></td>
<td>14.2</td>
<td>2.7</td>
</tr>
<tr>
<td><strong>Eggs with oil globule</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Turbot</td>
<td>40.0</td>
<td></td>
<td>29.0</td>
<td>23.0</td>
</tr>
<tr>
<td>Rainbow trout</td>
<td>49.7</td>
<td></td>
<td>46.8</td>
<td>0.2</td>
</tr>
<tr>
<td></td>
<td>37.3</td>
<td>62.8</td>
<td>56.5</td>
<td>0</td>
</tr>
<tr>
<td>Japanese eel</td>
<td>19.4</td>
<td>80.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Walleye</td>
<td>21.8</td>
<td>78.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Burbot</td>
<td>12.6</td>
<td></td>
<td>4.1</td>
<td>81.8</td>
</tr>
<tr>
<td>Striped bass</td>
<td>11.0</td>
<td></td>
<td>11.0</td>
<td>79.0</td>
</tr>
<tr>
<td>Whitefish (<em>Coregonus sp.</em>)</td>
<td>31.7</td>
<td></td>
<td>64.9</td>
<td>1.8</td>
</tr>
<tr>
<td><em>Perca fluviatilis</em></td>
<td>13.5 – 17.3</td>
<td>82.4 - 86.5</td>
<td>8.1 - 12.4</td>
<td>68.0 – 68.9</td>
</tr>
<tr>
<td>(Eurasian perch)</td>
<td>14.3</td>
<td></td>
<td>1.1</td>
<td>83.7</td>
</tr>
<tr>
<td><em>P. flavescens</em></td>
<td></td>
<td>2.8</td>
<td>77.8</td>
<td></td>
</tr>
<tr>
<td>(yellow perch)</td>
<td></td>
<td></td>
<td></td>
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</tbody>
</table>

Linoleic acid 18:2n6

Domestic females:
Declining fatty acid as winter duration increases

![Graph showing the relationship between winter duration and linoleic acid 18:2n6. The graph indicates a decline in linoleic acid as winter duration increases, with a P value of 0.07 and an R^2 value of 0.19.](image)
Linoleic acid 18:2n6

Erie females:
Declining hatching success as fatty acid increases
Linolenic acid 18:3n3

Domestic females:
Declining fatty acid as winter duration increases

![Graph showing the relationship between winter duration and 18:3 fatty acid content.](image)

- **Domestic**
  - $p = 0.09$
  - $R^2 = 0.16$
Linolenic 18:3n3

Erie females:
Declining hatching success as fatty acid increases

![Graph showing the relationship between Linolenic 18:3n3 and hatching success.](image)

- **Erie**
  - $P = 0.004$
  - $R^2 = 0.39$

- Non-injected
- Injected

- Proportion Hatched vs. 18:3 (% by weight)
“Mead” acid 20:3n9?

Domestic females:
\[ \uparrow \text{Length} = \uparrow \text{Fatty acid} \]

Erie females:
\[ \uparrow \text{Fatty acid} = \downarrow \text{Hatching success} \]
C 22:0 (saturated)

Domestic females:
↑ Fatty acid = ↑ Hatching success

Erie females:
↑ Fatty acid = ↓ Hatching success

- Domestic
  - P = 0.013
  - R² = 0.20

- Erie
  - P = 0.07
Docosahexaenoic (DHA) 22:6n3

Domestic females:
Declining essential fatty acid as winter duration increases
DHA C22:6n3

Erie females:
Declining hatching success as DHA fatty acid increases
Conclusions
Extended period of low water temperatures results in increased sum of degree-days, thermal exposure at low temperatures enhances stress on membranes integrity and lipid oxidative cascade is initiated.

Docosahexaenoic acid (C22:6n3)
Essential PUFA

B.J. Lee in lake trout embryos at 1-2°C
Ovulated, hydrated (unfertilized) and freeze-dried eggs of yellow perch. Domesticated, fed commercial (Aquamax) diet. Collected in June 2013 (n=6 females)
Recovery of the lipid classes after separation of NL fraction of yellow perch egg lipids on ion-exchange column

<table>
<thead>
<tr>
<th></th>
<th>Total load [g]</th>
<th>WE1 [%]</th>
<th>WE2 [%]</th>
<th>TAG [%]</th>
<th>PL [%]</th>
<th>Recovery rate [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>LE YP eggs</td>
<td>0.300</td>
<td>74.64</td>
<td>4.64</td>
<td>3.33</td>
<td>1.48</td>
<td>84.09</td>
</tr>
<tr>
<td>LE YP eggs 2</td>
<td>0.315</td>
<td>75.96</td>
<td>3.18</td>
<td>3.71</td>
<td>1.55</td>
<td>84.40</td>
</tr>
<tr>
<td>Ovulated YP eggs</td>
<td>0.330</td>
<td>64.93</td>
<td>8.79</td>
<td>1.74</td>
<td>1.43</td>
<td>76.89</td>
</tr>
<tr>
<td>Fatty acid composition of neutral (NL) and polar (PL) fraction of yellow perch eggs</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>-----------------------------------------------</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td><strong>Fatty acid</strong></td>
<td><strong>NL fraction</strong></td>
<td><strong>PL fraction</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C14:0</td>
<td>0.40</td>
<td>2.43</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C14:1</td>
<td>0.14</td>
<td>1.05</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C16:0</td>
<td>5.16</td>
<td>29.05</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C16:1[n-9]</td>
<td>23.51</td>
<td>8.27</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C18:0</td>
<td>0.01</td>
<td>0.04</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C18:1[n-9c]</td>
<td>25.27</td>
<td>12.98</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C18:1[n-7]</td>
<td>2.35</td>
<td>4.46</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C18:2[n-6c]</td>
<td>1.12</td>
<td>2.71</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C18:3[n-3]</td>
<td>0.33</td>
<td>0.26</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C20:0 #1</td>
<td>0.46</td>
<td>0.11</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C20:1[n-9]</td>
<td>0.40</td>
<td>0.25</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C20:2[n-6]</td>
<td>0.42</td>
<td>2.77</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C20:4[n-6]</td>
<td>0.03</td>
<td>1.14</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C20:3[n-3]</td>
<td>0.09</td>
<td>0.25</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C20:5[n-3]</td>
<td>9.22</td>
<td>9.64</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C22:0</td>
<td>0.94</td>
<td>0.21</td>
<td></td>
<td></td>
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<tr>
<td>C22:1</td>
<td>0.35</td>
<td>0.63</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C22:6[n-3]</td>
<td>29.79</td>
<td>23.76</td>
<td></td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>
Fatty acid profiles of different lipid classes in non-ovulated, matured eggs of yellow perch (GSI 22-28%) – 2
Fatty acid profiles of different lipid classes in non-ovulated, matured eggs of yellow perch (GSI 22-28%) – 1
Uptake, transport and metabolism of lipids in the life cycle of yellow perch

**Gut**
- Diets provide NL and PL
- Hydrolysis
- No waxes!

**Liver**
- Synthesis of vitellogenin
- No waxes!

**Muscle**
- Synthesis of NL and PL
- No waxes!

**Ovary**
- Synthesis of waxes

**Embryo/Larvae**
- Utilization of waxes
- Synthesis of PL and NL
Final conclusions

1. Short winter for gametogenesis does not mean bad for reproduction

2. Short winter means good for essential fatty acid profiles in eggs/embryos

3. Long period of exposure in low temperatures can mean higher utilization rate (loss) of DHA (PUFA) to protect cell membranes

4. Long winter may mean higher demand for antioxidants (vitamins)!
5. Lipid and fatty acid profiles in ovulated eggs show great promise in predicting viability of embryos and (perhaps) larvae.

6. Extensive transformation of lipid classes takes place following ovulation, endogenous and mixed feeding phases.

7. Wax esters may provide advantages in respect to water/ion balance, microbial detachment, swim bladder inflation in early life of freshwater fish.

8. Enrichment of the first larval diets with specific wax esters should be examined.
Acknowledgements

OSU
John Grayson
Karolina Kwasek
Tim Parker
Jeramy Pinkerton
Theo Gover
Chelsea Schmit
AEL Students & Staff

ODOW - Fairport
Kevin Kayle
Ann Marie Gorman
Carey Knight
John Deller
Bob Bennett
Sherr Vue
Nick Agins

ODOW - Sandusky
Jeff Tyson
Chris Vandergoot
Mark Turner
Travis Hartman
Eric Weimer
Jim McFee

USGS – LEBs
Richard Kraus
Patrick Kocovsky
Dale Hall
Tim Cherry
Brandon Giesler

Ohio Sea Grant
John Hageman
Matt Thomas
Fatty acid concentration (g/100 g lipid class)

- C14:0
- C16:0
- C16:1
- C18:1

Saturated

Unsaturated
Fatty acid concentration (g/100 g lipid class)

- C18:2n6
- C18:3n3
- ARA
- EPA
- DHA
- n3/n6
Effect of lipid and fatty acid composition on embryo viability of Japanese eel (*Anguilla japonica*) (Furuita et al. 2006. J. Fish Biol. 69: 1178)
Results on a per egg basis

Example using caloric density:

Energy density declines as winter duration increases
Lipid classes and fatty acid composition of perch food (fathead minnows) offered in the course of gametogenesis 2011-2012

<table>
<thead>
<tr>
<th>Category</th>
<th>Mean % ± StdErr</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Lipids</td>
<td>3.5 % ± 0.3</td>
</tr>
<tr>
<td>Neutral Lipids</td>
<td>65 % ± 2.5</td>
</tr>
<tr>
<td>Phospholipids</td>
<td>35 % ± 2.4</td>
</tr>
<tr>
<td>Linoleic C18:2 (n6)</td>
<td>0.04 % ± 0.01</td>
</tr>
<tr>
<td>Linolenic C18:3 (n3)</td>
<td>0.40 % ± 0.07</td>
</tr>
<tr>
<td>Arachidonic C20:5 (n3)</td>
<td>5.4 % ± 1.4</td>
</tr>
<tr>
<td>EPA C20:4 (n6)</td>
<td>0.02 % ± 0.09</td>
</tr>
<tr>
<td>DHA C22:6 (n3)</td>
<td>5.5 % ± 1.7</td>
</tr>
<tr>
<td>n3 /n6</td>
<td>8.3 : 1.0</td>
</tr>
</tbody>
</table>
Recovery of the lipid classes after separation of NL fraction of yellow perch egg lipids on ion-exchange column

<table>
<thead>
<tr>
<th></th>
<th>WE1</th>
<th>WE2</th>
<th>TAG</th>
<th>PE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>64.93%</td>
<td>75.30%</td>
<td>8.79</td>
<td>3.91</td>
</tr>
<tr>
<td></td>
<td>8.79%</td>
<td>3.91%</td>
<td>1.74</td>
<td>1.52</td>
</tr>
<tr>
<td></td>
<td>1.74%</td>
<td>3.52%</td>
<td>1.43</td>
<td>1.52</td>
</tr>
</tbody>
</table>

LE YP eggs | spawned domestic YP eggs
Lipids recovery after ion-exchange separation of NL from yellow perch eggs

LE YP eggs: 76.89%
Spawning domestic YP eggs: 84.25%
Cumulative water temperature from initiation of gametogenesis (October) to ovulation (degree-days, °D)

<table>
<thead>
<tr>
<th>Origin</th>
<th>Erie</th>
<th>Domestic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Winter duration</td>
<td>Short</td>
<td>Long</td>
</tr>
<tr>
<td>Degree-days</td>
<td>2,158</td>
<td>1,771</td>
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<tr>
<td>Difference</td>
<td>18%</td>
<td></td>
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</tbody>
</table>
YP eggs Troy’s

C22:6[n-3]

<table>
<thead>
<tr>
<th>Category</th>
<th>Value</th>
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</thead>
<tbody>
<tr>
<td>WE total</td>
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<tr>
<td>WE I FFA</td>
<td>41.46</td>
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<td>WE II</td>
<td>6.48</td>
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<td>WE1</td>
<td>28.32</td>
</tr>
<tr>
<td>WE2</td>
<td>23.02</td>
</tr>
</tbody>
</table>
Results on a per egg basis

Example using caloric density:

*Hatching success declines with increasing energy density*
Results on a per egg basis

But when results are scaled to calories per egg:

- **Hatching success increases as the calories per egg increase.**
- **This is because eggs/g (of ribbon) also decreased with increasing winter duration.**
- **Even though the density declines, fewer eggs/g translates into higher calories per egg.**
Time of divergence among teleost fishes
(Saito et al. 2011. PLOS)