Marker molecolari per il monitoraggio del benessere animale in acquacoltura

Dipartimento di Biotecnologie e Scienze Molecolari

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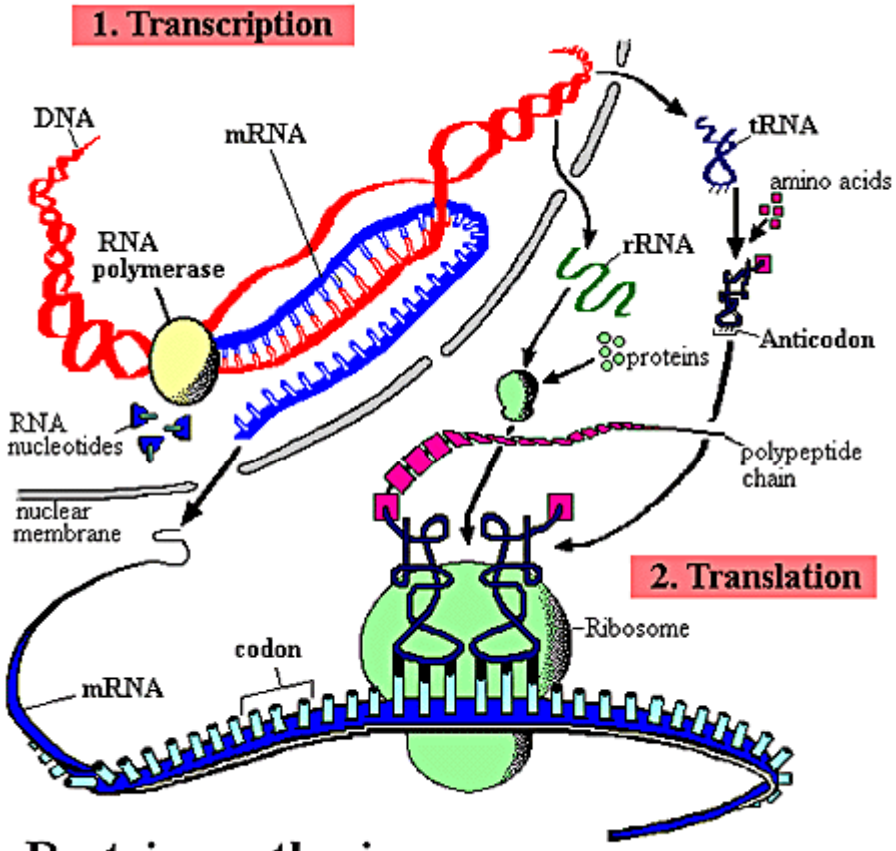
A SEARCH FOR BIOMARKERS

In the past, biomarkers were primarily physiological indicators such as blood pressure or heart rate. More recently, biomarker has drifted toward being a synonym for **molecular biomarker**, such as elevated prostate specific antigen as a molecular biomarker for prostate cancer.

Molecular biomarkers are an **early** sign of change in an organism's physiological state - such as adaptation, stress or injury - due to environmental factors or disease. Changes in molecules such as these (in our case mRNAs) are sensitive and specific, making them useful sentinels of an organism's exposure to a specific environmental agent.



Protein synthesis

MKTFSVAVAVAVVLTFICIQQSSA
VPATEVQELEEPMGIENLAAEHEE
TSVDSWKMPYNNRHKRGFKCRVCC
GCCTPGVCGLCCRF

5′–CCTCGTGCCGAATTCGGCAC
GAGGAAGAGCTGACGGAGACACCTAAAGGTCTGAAG
AAGTCTACTTGATTGAACAGTTTGAACCGTCCTAAG
ATGAAGACATTCAGTGTTGCAGTTGCAGTGGCCGTC
GTGCTCACCTTTATTTGTATTCAGCAGAGCTCTGCT
GTCCCAGCCACTGAAGTGCAAGAGCTGGAGGAGCCA
ATGGGCATTGAGAATCTGGCTGCTGAACATGAGGAG
ACATCAGTGGACTCGTGGAAGATGCCGTATAACAAC
AGACACAAGCGTGGCTTTAAGTGTCGCGTTTGCTGC
GGCTGCTGCACCCCCGGTGTCTGCGGATTGTGCTGC
AGATTC**TGA***GGATTCCTGCTCCAACAACTACTGTAC*
AATTCAGATGTTTCTAAACTTCATCATGCTGTAGTT
AATGTGCATGTACTCAGTGATGTGATGCATCGTCCA
CTGGTTTTGCTAAATATCTGATACTGCTGTGGATTG
TCAC<u>AATAAA</u>GTTGAATGTCACTAAAAAAAAAAAA

we can use two different approaches

1- To use genes known to be modified in their expression by the treatment

2- To find genes differentially expressed in two mRNA pools by Differential Display (DD) or subtractive libraries

-1st approach-

Select a panel of genes of interest (e.g., HSPs, CYPs and MTs)

Search in gene databases these sequences for the species of interest

If these sequences are present in public databases: design primers for PCR analysis

If not: try something to obtain your primers and ... good luck!

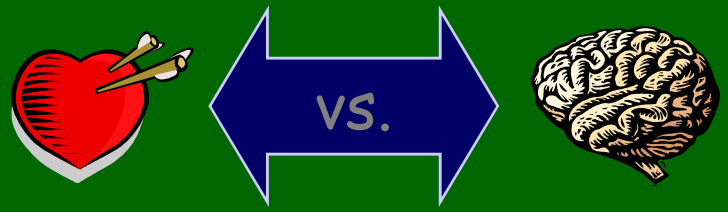
RNA extraction, retrotranscription, PCR with the designed primers and product analysis (gel) or real-time PCR

-2nd approach-

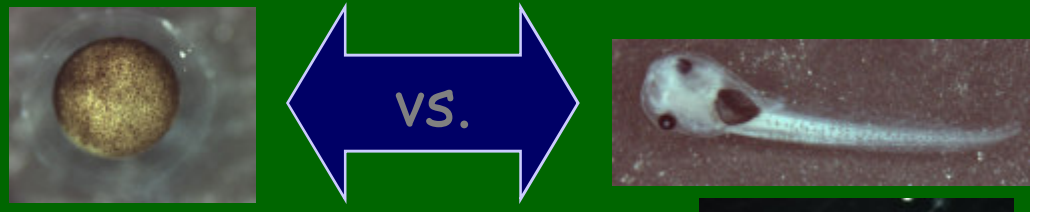
DIFFERENTIAL DISPLAY

Comparison of two of more mRNA pools

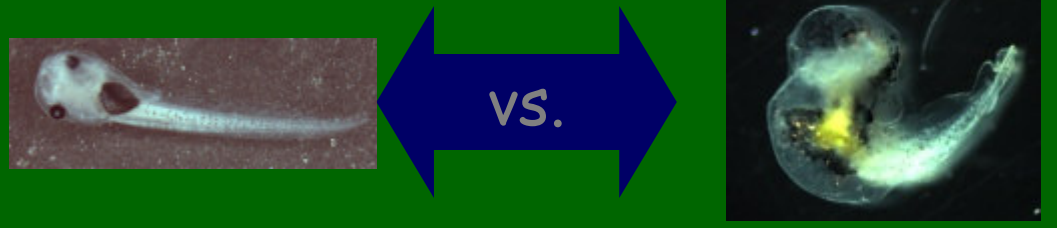
Tissues

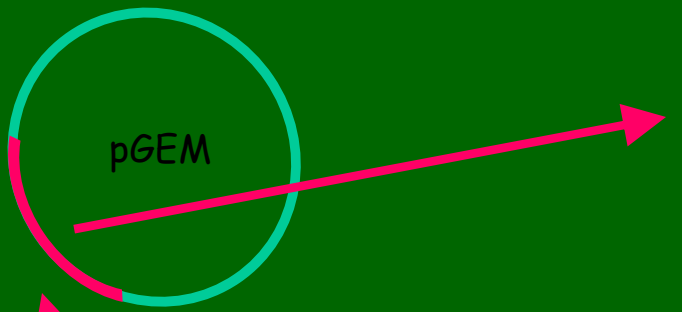


Developmental stages



Control and treated samples





AAGCTTTGAGACTTGAACATACCCCGTCTTGACTTGGAATTTGACTAGAGACTAG
AGCAGAACATTCTTGTATTCTGAATCTCTCACATTTTTCCTGAAGTTGTTAGTCG
TTATTTCACCTAAATGAATATCACATGTTCACGATGAGGTGTGCTTTTGTGAGAT
TTGATCATTAGCATAACTGACTCCCAAATCGCTATATTATGCTGCCTCTTCCATT
GTCTGCGGGCACGGTACATTCATAAGAATCTTACCAAAGATTTAAAAGAAGACTC
TGTAGTGGTGTTAGAGGACCTGAAACAATTGGAAATTATTATTATTTCATCAAAG
AACCATCTACATGAAACATCTTTCTAAAGCAAAGCGGTCCATGACTTTGATGGGA
GGAGGTCAAGAAAATGTGAAATTTAAGAGTAGTTAATTGTATTGACTATCAAAGG
TGCACATTCATTTAAATAATGGCATACTTCTTATTTCAAACATGTTTTTCCCTGT
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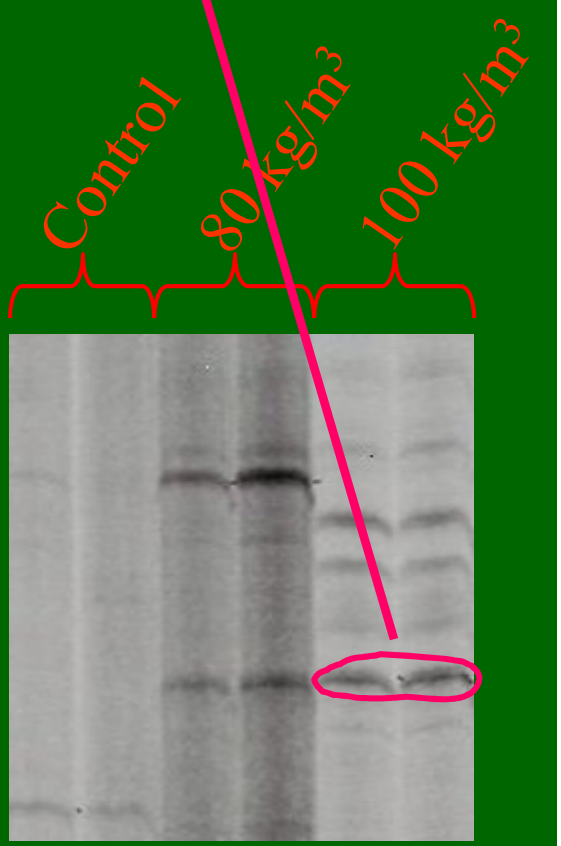
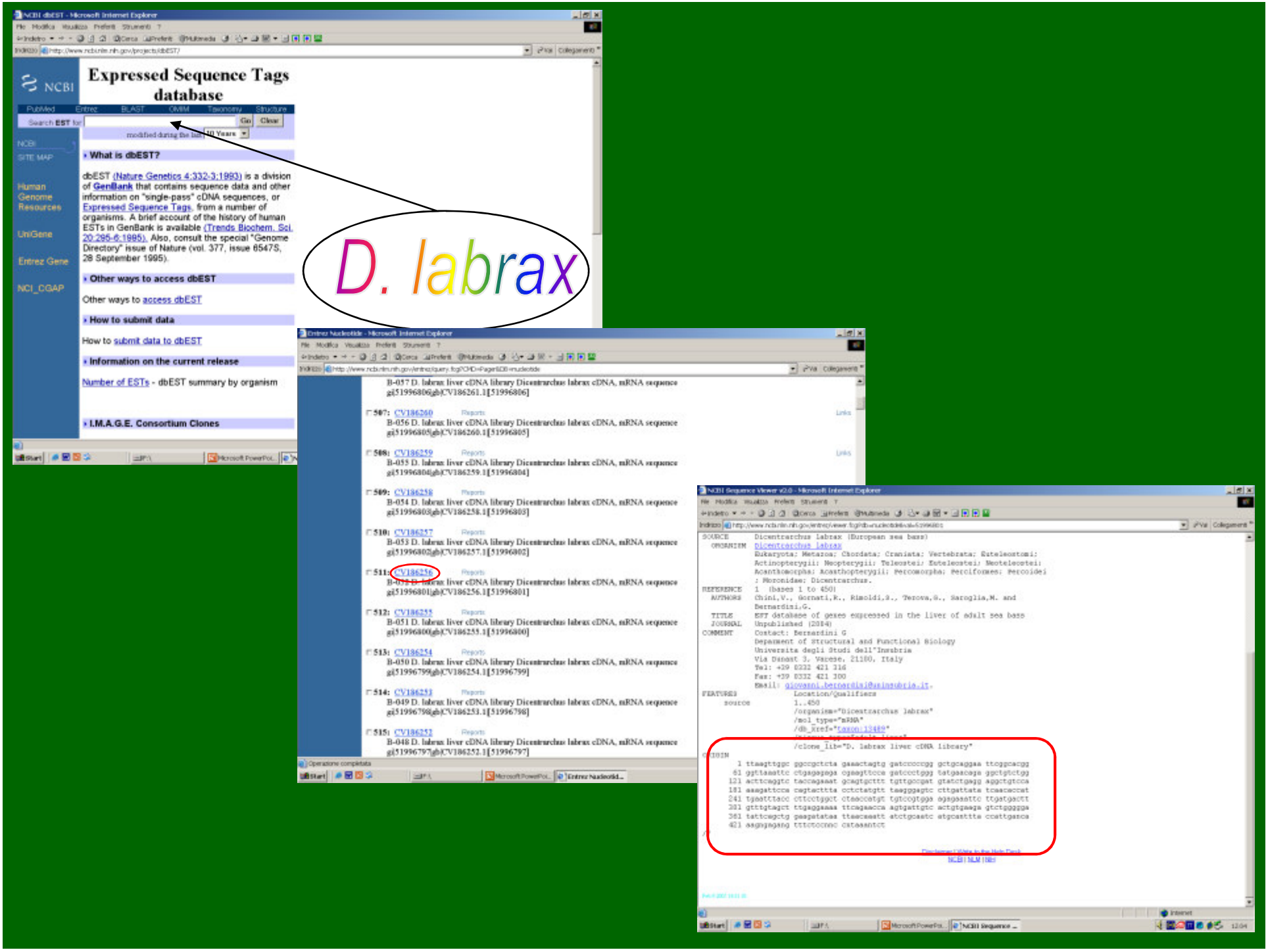


BAND		EXPRESSION			HOMOLOGY
NAME	LENGTH	CONTROL	80kg/m^3	100kg/m^3	
1P49	193 pb	+	+++	+++	
5P49	219 pb	+++	+	+	
6P49	200 pb	+	+++	+++	
8P49	239 pb	+++	+	+	
1P50	588 pb	+++	+	+	
3P50	400 pb	+	+++	+++	
4P50	289 pb	+++	+	+	
5P50	186 pb	+	+++	+++	
6P50	172 pb	+	+++	+++	
7P50	501 pb	+++	+	+	
8P50	263 pb	+++	+	+	U5 small ribonucleoprotein Mus musculus
11P50	295 pb	+	+++	+++	

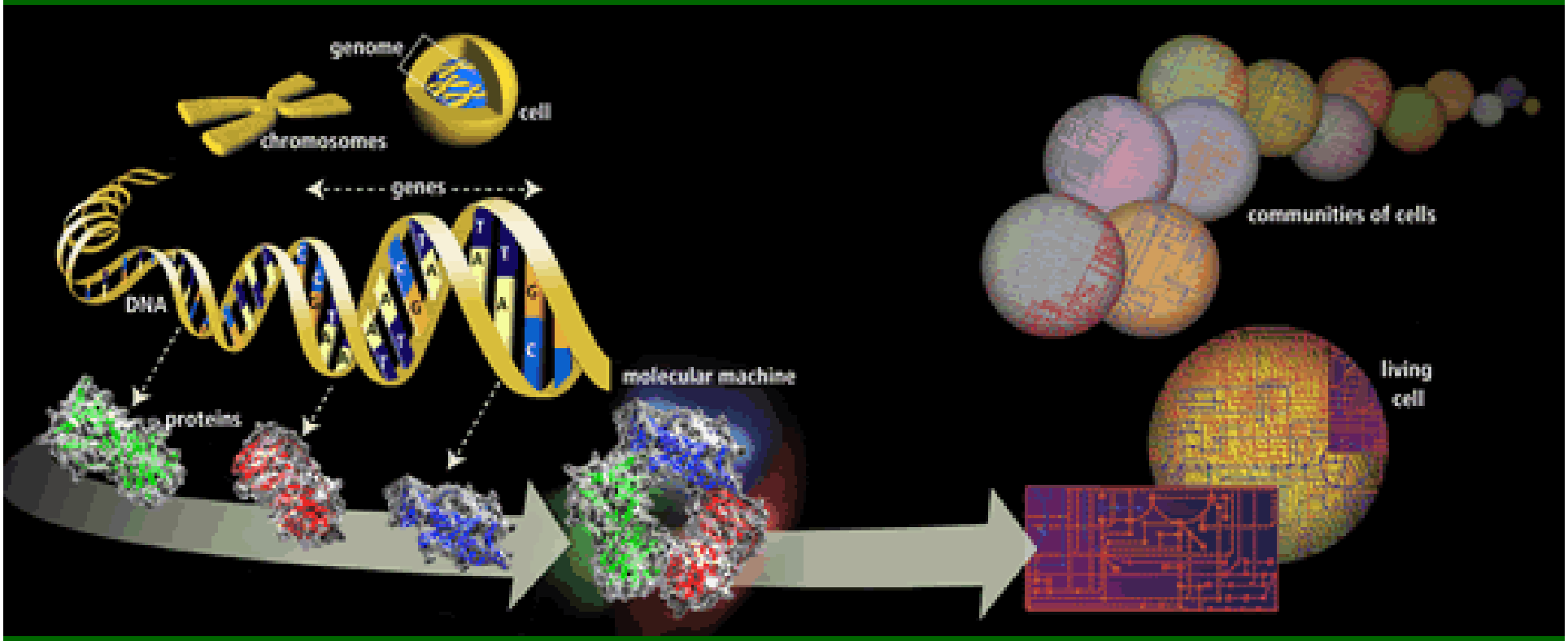
PUNCTUM DOLENS !!!

No molecular information on species that

are not "*model organisms*" notwithstanding a great commercial interest


Expressed Sequence Tags database
What is dbEST?
Other ways to access dbEST
How to submit data
Information on the current release
I.M.A.G.E. Consortium Clones
D. labrax

E quindi l'approccio molecolare ci permette di "chiedere" al pesce: benessere, condizione di ossigenazione, trend di crescita, maturazione sessuale, ambiente inquinato, …

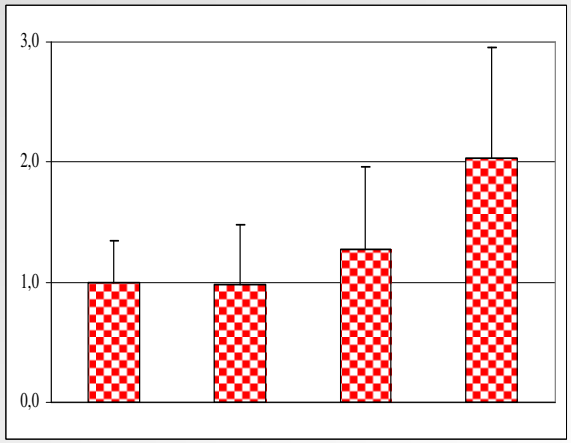


…e di ricevere delle risposte!

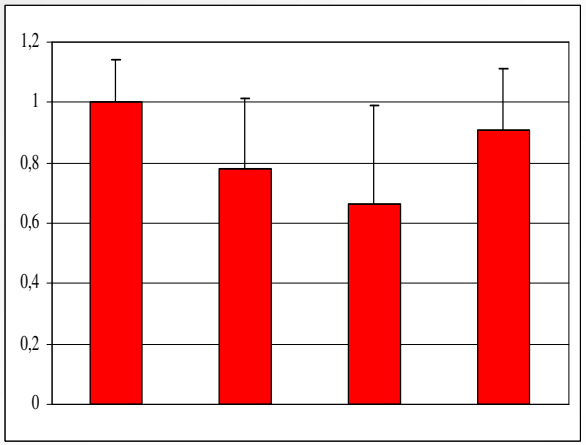
ESEMPLIFICHIAMO…

- Come stai? HSP70, HSP90, HMGCoA reduttasi, BDNF,…
- Ci sono infezioni in giro? Hepcidin
- Cresci bene? Miostatina, miosina, fattori di crescita,…
- Hai appetito? Ghrelin
- Digerisci bene? Gastricsin
- Hai avuto caldo? WAP

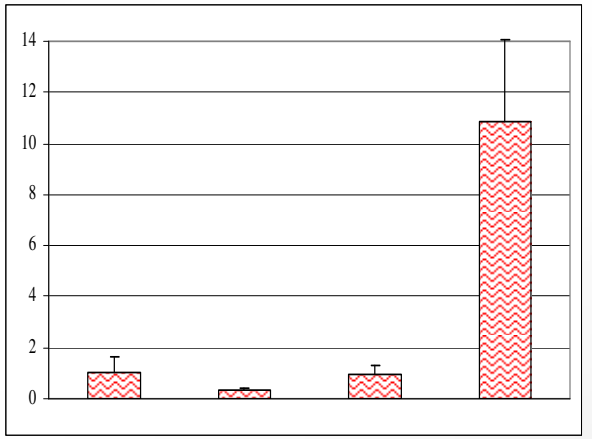
Real time PCR_1 DENSITA' DI POPOLAZIONE



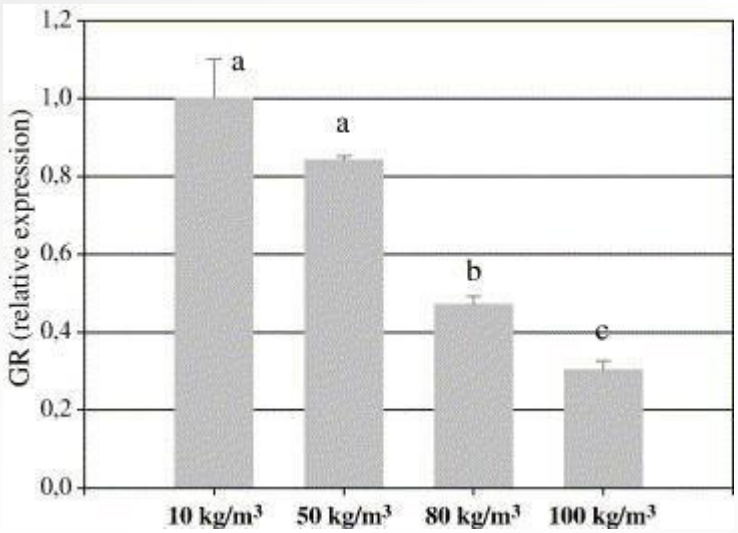
HSP70



HSP90



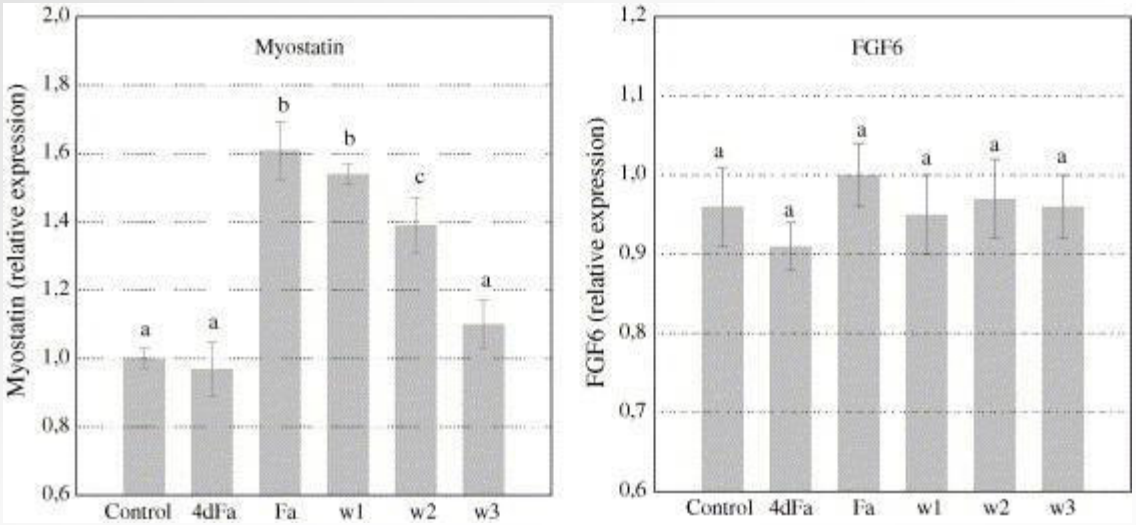
HMGCR



GR

D.labrax liver reared at 10, 50, 80, 100 kg/m^3
n=3. Cytoskeletal actin has been used as endogenous control

Real time PCR_2 DIGIUNO

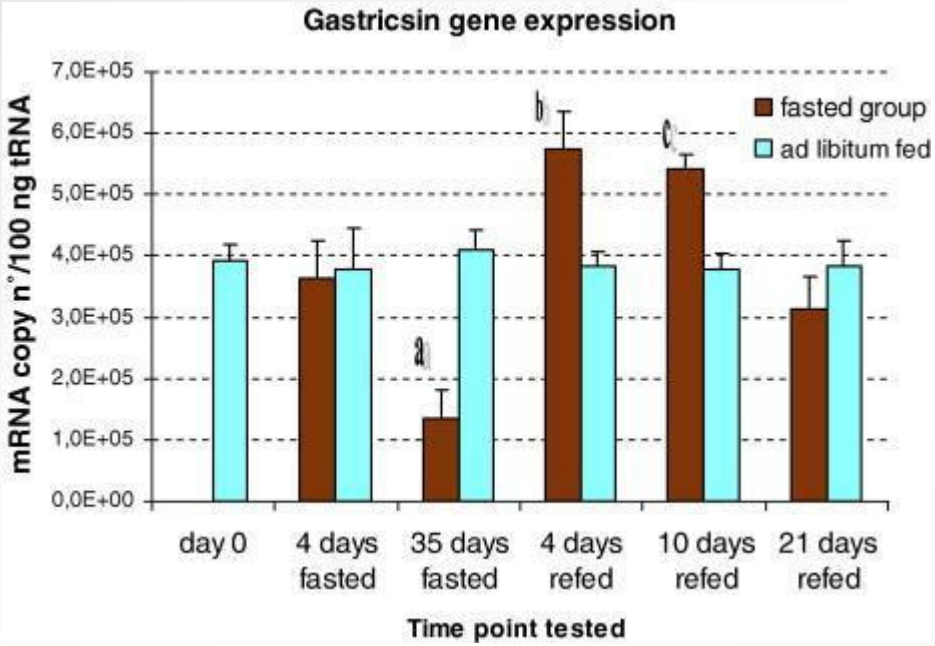


Myostatin and FGF6 mRNA expression in *D. labrax* muscle. Cytoskeletal actin has been used as endogenous control. Fish were sampled before fasting

Control: prima del digiuno
4dFa: 4 giorni dopo il digiuno
Fa: dopo 4 settimane di digiuno
w1, w2, w3: 1, 2, 3 settimane dopo la rialimentazione

n=3 Bars indicate standard error of the mean. Differences were determined by one-way analysis of variance (ANOVA). Differences between letters indicate significantly different means ($P < 0.01$).

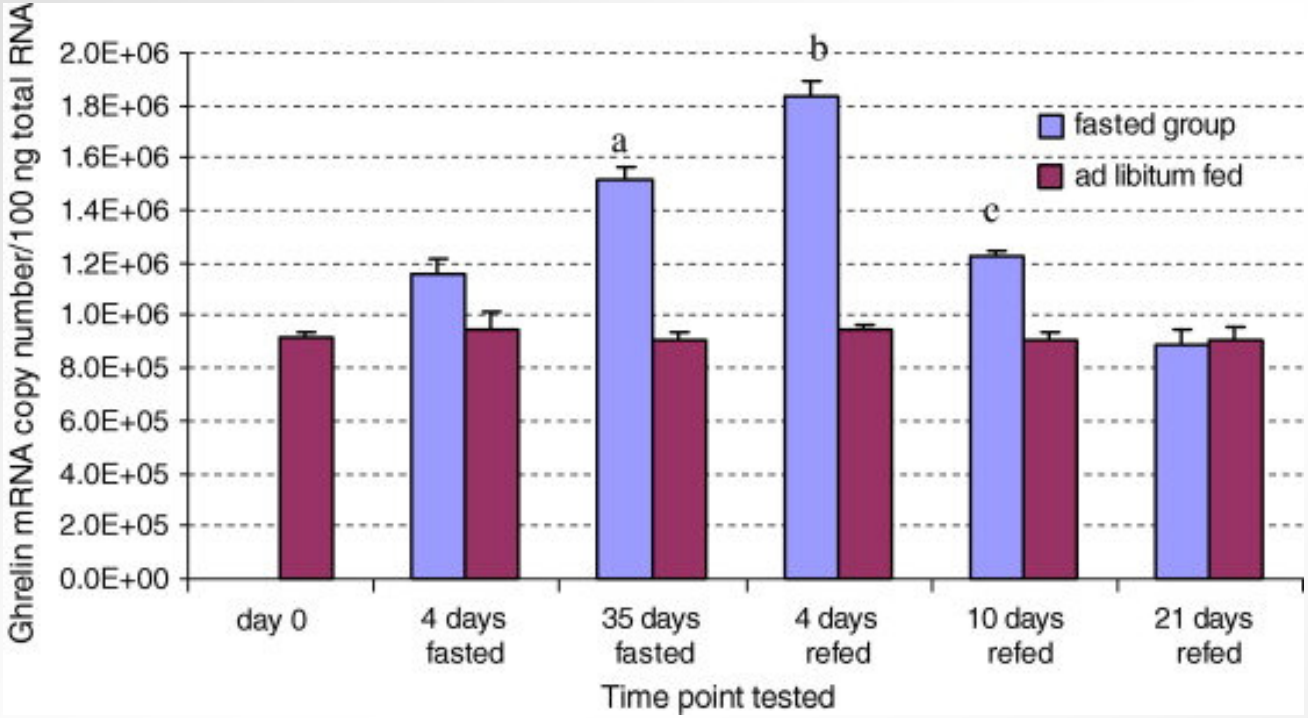
Real time PCR_3 DIGIUNO



Pepsinogen C mRNA in *D. labrax* stomach in the course of the experiment.

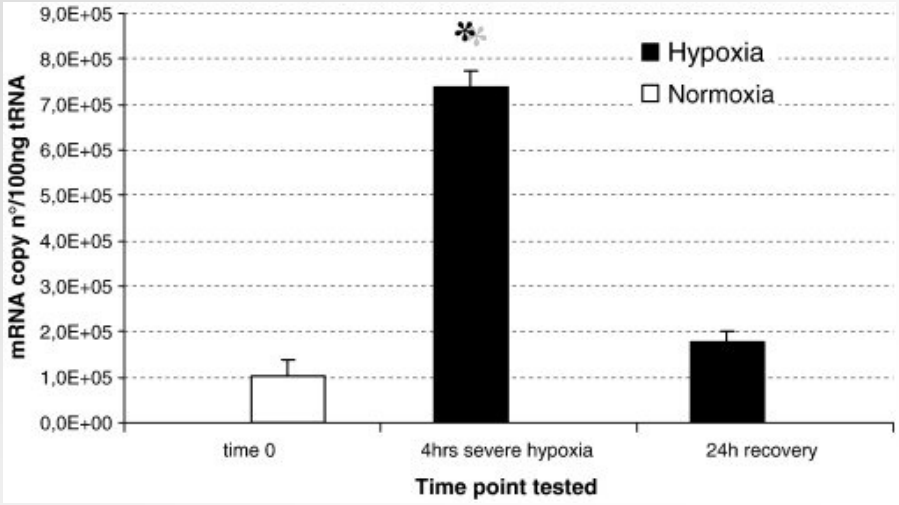
day 0: before fasting
4 days fasted: 4 giorni dopo il digiuno
35 days fasted: dopo 5 settimane di digiuno 4, 10 e 21 giorni dopo la rialimentazione.
n=5 ($P < 0.05$).

Real time PCR_4 DIGIUNO

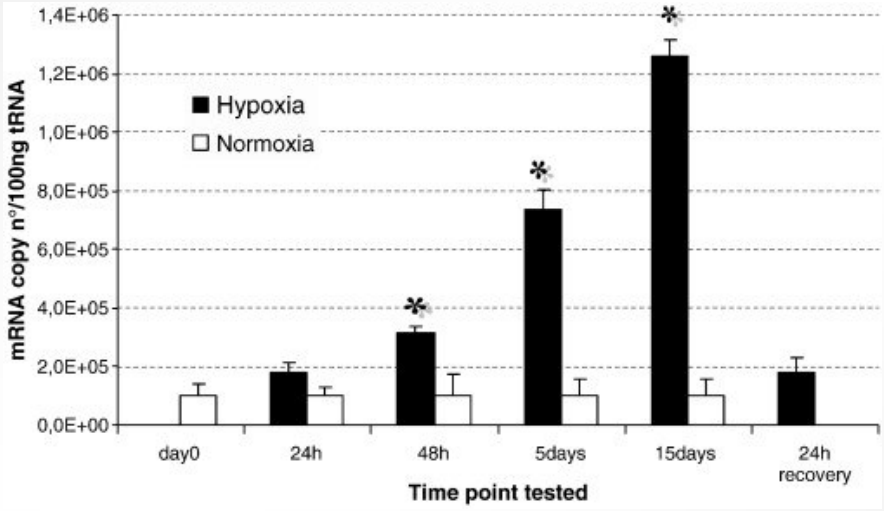


Ghrelin mRNA in D. labrax stomach
day 0: before fasting
4 days fasted: 4 giorni dopo il digiuno
35 days fasted: dopo 5 settimane di digiuno
4, 14 e 21 giorni dopo la rialimentazione.
n=5 ($P < 0.05$).
Non è chiaro se la grelina agisca direttamente su GH o su altri fattori ipotalamici collegati al comportamento alimentare

Real time PCR_5 IPOSSIA ACUTA E CRONICA



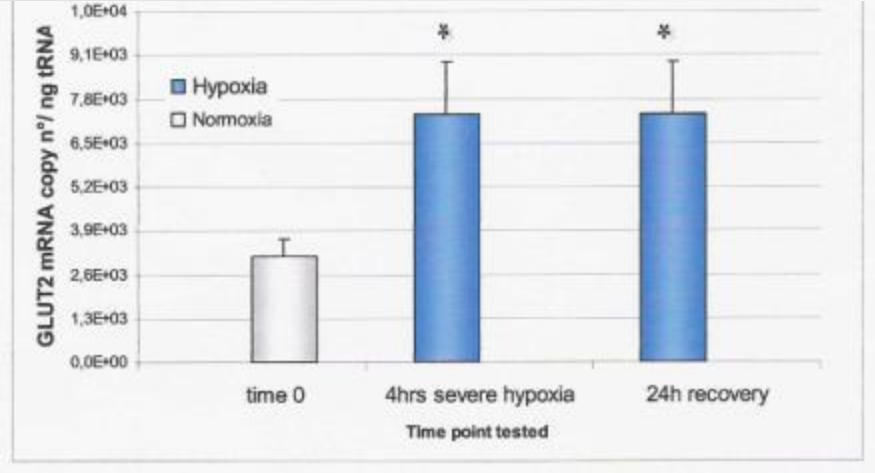
HIF-1α mRNA in *D. labrax* liver in the course of the acute-hypoxia exposure. Fish were sampled after continuous exposure for 4 h, to severe hypoxia conditions (D.O. 25%). After 4 h of hypoxia the DO levels were adjusted to normoxia and fish were sampled after 24 h of recovery.



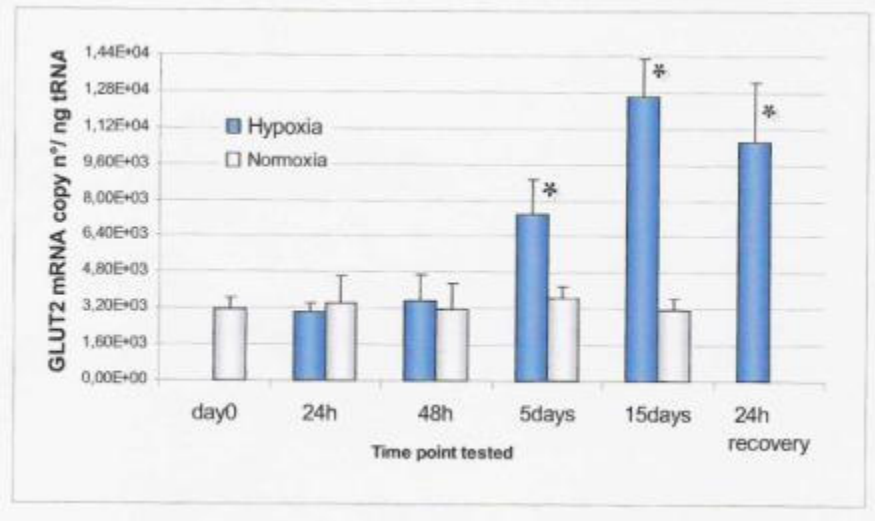
HIF-1α mRNA in *D. labrax* liver in the course of the chronic hypoxia exposure (D.O. 51% of saturation). Fish were sampled at the start of the experiment (day 0), and then after continuous exposure for 24 h, 48 h, 5, and 15 days to the hypoxia conditions. After 15 days of hypoxia the DO levels were adjusted to normoxia and fish were sampled after 24 h of recovery.

n=5 ($P < 0.05$)

Real time PCR_6 IPOSSIA



GLUT2 mRNA in *D. labrax* liver in the course of the acute-hypoxia exposure. Fish were sampled after continuous exposure for 4 h, to severe hypoxia conditions (D.O. 25% of saturation). After 4 h of hypoxia the DO levels were adjusted to normoxia and fish were sampled after 24 h of recovery.



GLUT2 mRNA in *D. labrax* liver in the course of the chronic hypoxia exposure (D.O. 51% of saturation). Fish were sampled at the start of the experiment (day 0), and then after continuous exposure for 24 h, 48 h, 5, and 15 days to the hypoxia conditions. After 15 days of hypoxia the D.O. levels were adjusted to normoxia and fish were sampled after 24 h of recovery.

n=5 ($P < 0.05$)

- Come stai? HSP70, HSP90, HMGCoA reduttasi, **BDNF**,…
- Ci sono infezioni in giro? Hepcidin
- Cresci bene? Miostatina, miosina, fattori di crescita,…
- Hai appetito? Ghrelin
- Digerisci bene? Gastricsin
- Hai avuto caldo? WAP

… e il pesce ci ha risposto!

Are BDNF levels correlated to an "anxiety" status in fish ?

- ***Science*** 10 February **2006**: Vol. 311. no. 5762, pp. 864 - 868
DOI: 10.1126/science.1120972
- **Essential Role of BDNF in the Mesolimbic Dopamine Pathway in Social Defeat Stress**
- *Olivier Berton, Colleen A. McClung, Ralph J. DiLeone, Vaishnav Krishnan, William Renthal, Scott J. Russo, Danielle Graham, Nadia M. Tsankova, Carlos A. Bolanos, Maribel Rios, Lisa M. Monteggia, David W. Self, Eric J. Nestler*
- **Mice experiencing repeated aggression develop a long-lasting aversion to social contact**, which can be normalized by chronic, but not acute, administration of antidepressant. Using viral-mediated, mesolimbic dopamine pathway–specific knockdown of brain-derived neurotrophic factor (BDNF), we showed that **BDNF is required for the development of this experience-dependent social aversion**. Gene profiling in the nucleus accumbens indicates that local knockdown of BDNF obliterates most of the effects of repeated aggression on gene expression within this circuit, with similar effects being produced by chronic treatment with antidepressant. These results establish an essential role for BDNF in mediating long-term neural and behavioral plasticity in response to aversive social experiences.

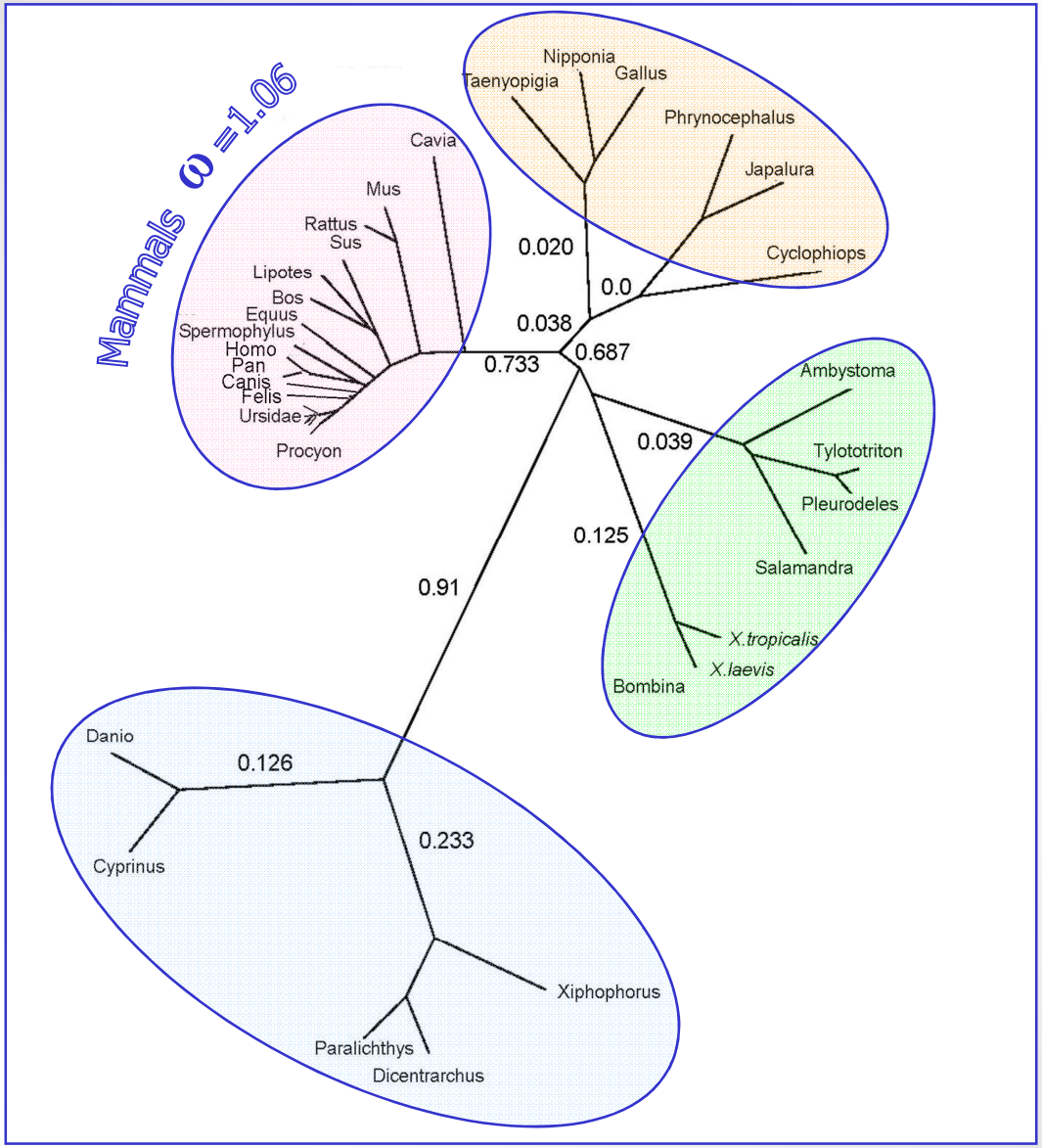
- *Nature Neuroscience* **10**, 1089 - 1093 (**2007**)
- **New insights into BDNF function in depression and anxiety**
- *Keri Martinowich, Husseini Manji & Bai Lu*
- The 'neurotrophin hypothesis of depression' is based largely on **correlations between stress or antidepressant treatment and down- or upregulation, respectively, of brain-derived neurotrophic factor (BDNF)**. Genetic disruption of the signaling pathways involving BDNF and its receptor, the tyrosine kinase TrkB, does not seem to cause depressive behaviors, but does hamper the effect of antidepressant drugs. Thus, BDNF may be a target of antidepressants, but not the sole mediator of depression or anxiety. Advances in BDNF cell biology, including its transcription through multiple promoters, trafficking and secretion, may provide new insights into its role in mood disorders. Moreover, **as the precursor proBDNF and the mature protein mBDNF can elicit opposite effects on cellular functions**, the impact of proBDNF and its cleavage on mood should be considered. Opposing influences of mBDNF and proBDNF on long-term potentiation and long-term depression might contribute to the dichotomy of BDNF actions on behaviors mediated by the brain stress and reward systems.

- **Psychoneuroendocrinology** Volume 34, Issue 6, July **2009**, Pages 833-843
- **Chronic stress increases pituitary adenylate cyclase-activating peptide (PACAP) and brain-derived neurotrophic factor (BDNF) mRNA expression in the bed nucleus of the stria terminalis (BNST): Roles for PACAP in anxiety-like behavior**
- *Sayamwong E. Hammack, Joseph Cheung, Kimberly M. Rhodes, Kristin C. Schutz, William A. Falls, Karen M. Braas and Victor May*
- Exposure to chronic stress has been argued to produce maladaptive anxiety-like behavioral states, and many of the brain regions associated with stressor responding also mediate anxiety-like behavior. Pituitary adenylate cyclase activating polypeptide (PACAP) and its specific G protein-coupled PAC1 receptor have been associated with many of these stress- and anxiety-associated brain regions, and signaling via this peptidergic system may facilitate the neuroplasticity associated with pathological affective states. **Here we investigated whether chronic stress increased transcript expression for** PACAP, PAC1 receptor, **brain-derived neurotrophic factor (BDNF), and tyrosine receptor kinase B (TrkB) in several nuclei.** In rats exposed to a 7 days chronic variate stress paradigm, chronic stress enhanced baseline startle responding induced by handling and exposure to bright lights. **Following chronic stress, quantitative transcript assessments of brain regions demonstrated dramatic increases in** PACAP and PAC1 receptor, **BDNF, and TrkB receptor mRNA expression** selectively in the dorsal aspect of the anterolateral bed nucleus of the stria terminalis (dBNST). Related vasoactive intestinal peptide (VIP) and VPAC receptor, and other stress peptide transcript levels were not altered compared to controls. Moreover, acute PACAP38 infusion into the dBNST resulted in a robust dose-dependent anxiogenic response on baseline startle responding that persisted for 7 days. PACAP/PAC1 receptor signaling has established trophic functions and its coordinate effects with chronic stress-induced dBNST BDNF and TrkB transcript expression may underlie the maladaptive BNST remodeling and plasticity **associated with anxiety-like behavior**.

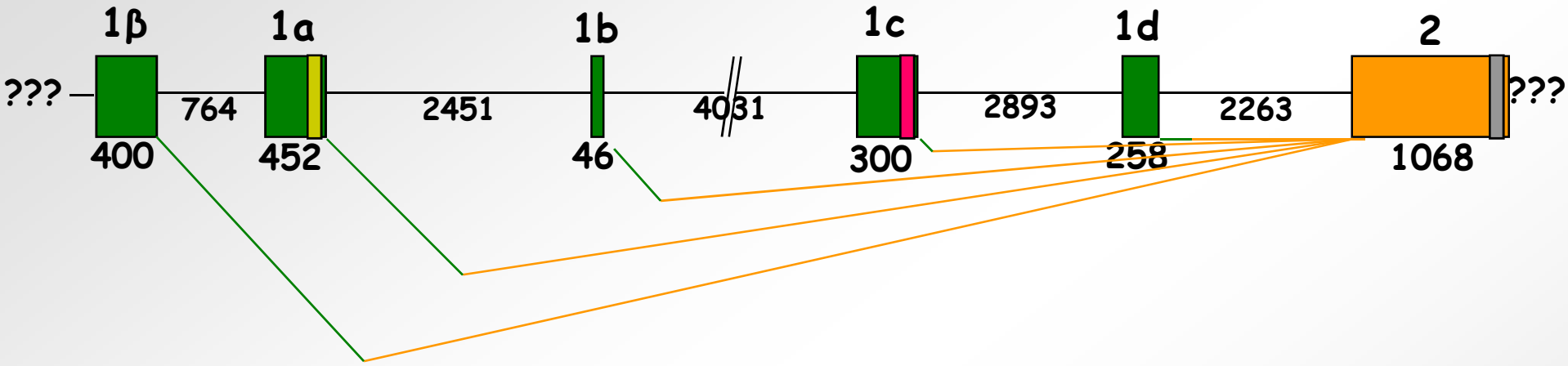

ANXIETY
BDNF

•**Neurotrophins** are a family of structurally related proteins required for the development and function of the vertebrate nervous system where they regulate several, and partially contrasting, aspects of the biology of neural cells, including survival, growth, differentiation, and cell death.

•Members of this wide family include Nerve Growth Factor (NGF) and **Brain-Derived Neurotrophic Factor (BDNF)**.


Mammals ω=1.06
Nipponia
Taenyopigia
Gallus
Phrynocephalus
Japalura
Cyclophiops
Cavia
Mus
Rattus
Sus
Lipotes
Bos
Equus
Spermophylus
Homo
Pan
Canis
Felis
Ursidae
Procyon
0.020
0.0
0.038
0.733
0.687
Ambystoma
0.039
Tylototriton
Pleurodeles
0.125
Salamandra
X.tropicalis
X.laevis
Bombina
0.91
Danio
0.126
Cyprinus
0.233
Xiphophorus
Paralichthys
Dicentrarchus

BDNF gene structure in D. labrax (FJ711591)



Exon	Start	Splice acceptor	End	Splice donor	Length (bp)
1β	nt 321	-	nt 602	GGAAAATGgtaagtag	282
1a	nt 1367	-	nt 1818	TTGTAAAGgtaagagc	452
1b	nt 4270	-	nt 4315	ACCTGATGgtaggttt	46
1c	nt 8347	-	nt 8646	AGTAAAAGgtatgtgt	300
1d	nt 11540	-	nt 11797	CTGTGGTTgttatgct	258
2	nt 14063	ccctccagTTCCACCA	nt 15130	-	1068

Highly Conserved Sequences

HCS1 (38-41%)

```
R.n./M.m.       ATTACCTCCGCCATGCAATTTCC---ACTATCAATAATTTAA 39
H.s.            ATTACCTCCGCCATGCAATTTCC---ACTATCAATAATTTAA 39
D.r.            ATTACCTCAACCATGCAATTTCC---ACCATCAATAATTTAA 39
D.l.            GCAGCCATGGGAGTGCATTACCTCATACCATCAATAATTTAA 42
                    **       **** *  *    ** ************
```

HCS2 (96%)

```
R.n./M.m        GTTAACTTTGGGAAATGCAAGTGTT 25
H.s.            GTTAACTTTGGGAAATGCAAGTGTT 25
D.r.            GTTAACTTTGGGAAATGCAAGTGTT 25
D.l.            GTTAACTTTGGGAAATGCAAGTCTT 25
                ********************** **
```

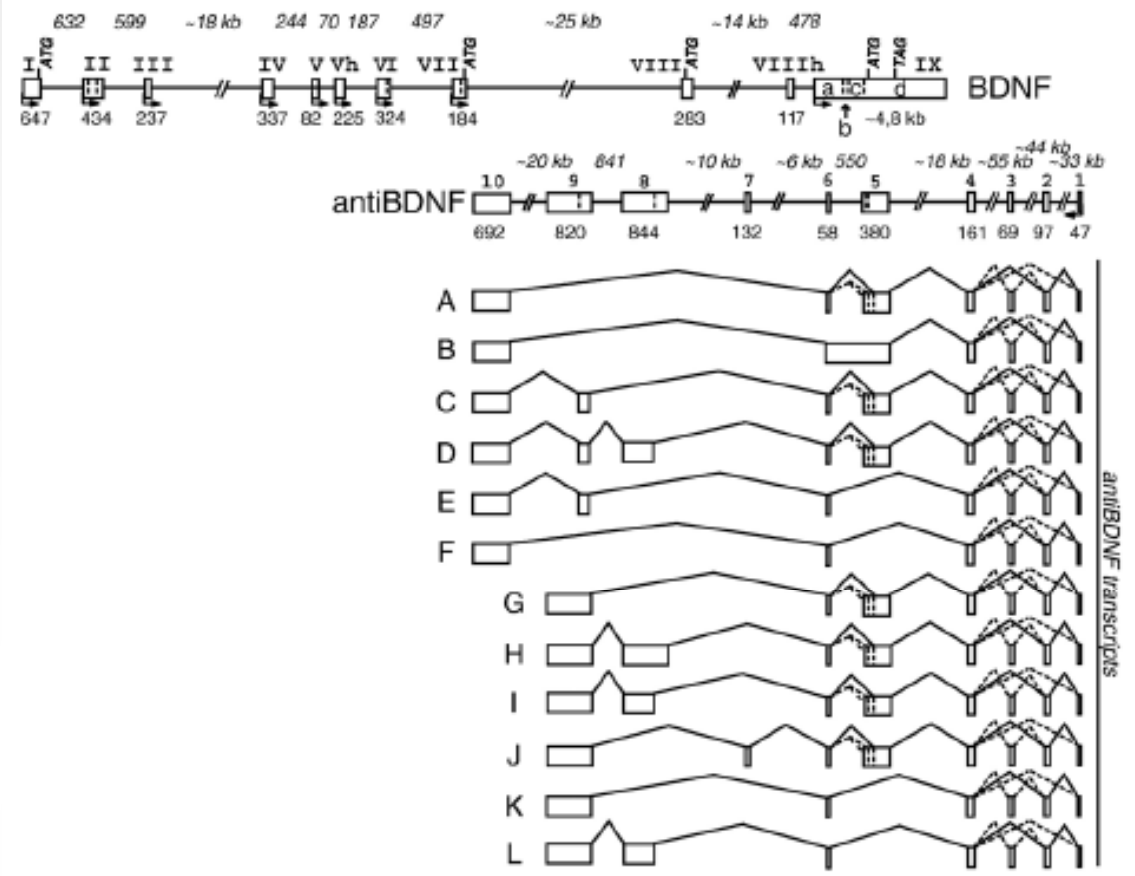
HCS3 (97%)

```
D.r.            TATCTATTTGTATAT--ACATAACAGGGTAAATTATTCAGT 39
H.s.            TATCTATTTGTATATATACATAACAGGGTAAATTATTCAGT 41
R.n./M.m        TATCTATTTGTATATATACATAACAGGGTAAATTATTCAGT 41
D.l.            TATCTATTTGTATATATACATAACAGGGTAAATTATTCCGT 41
                **************  ********************** **
```

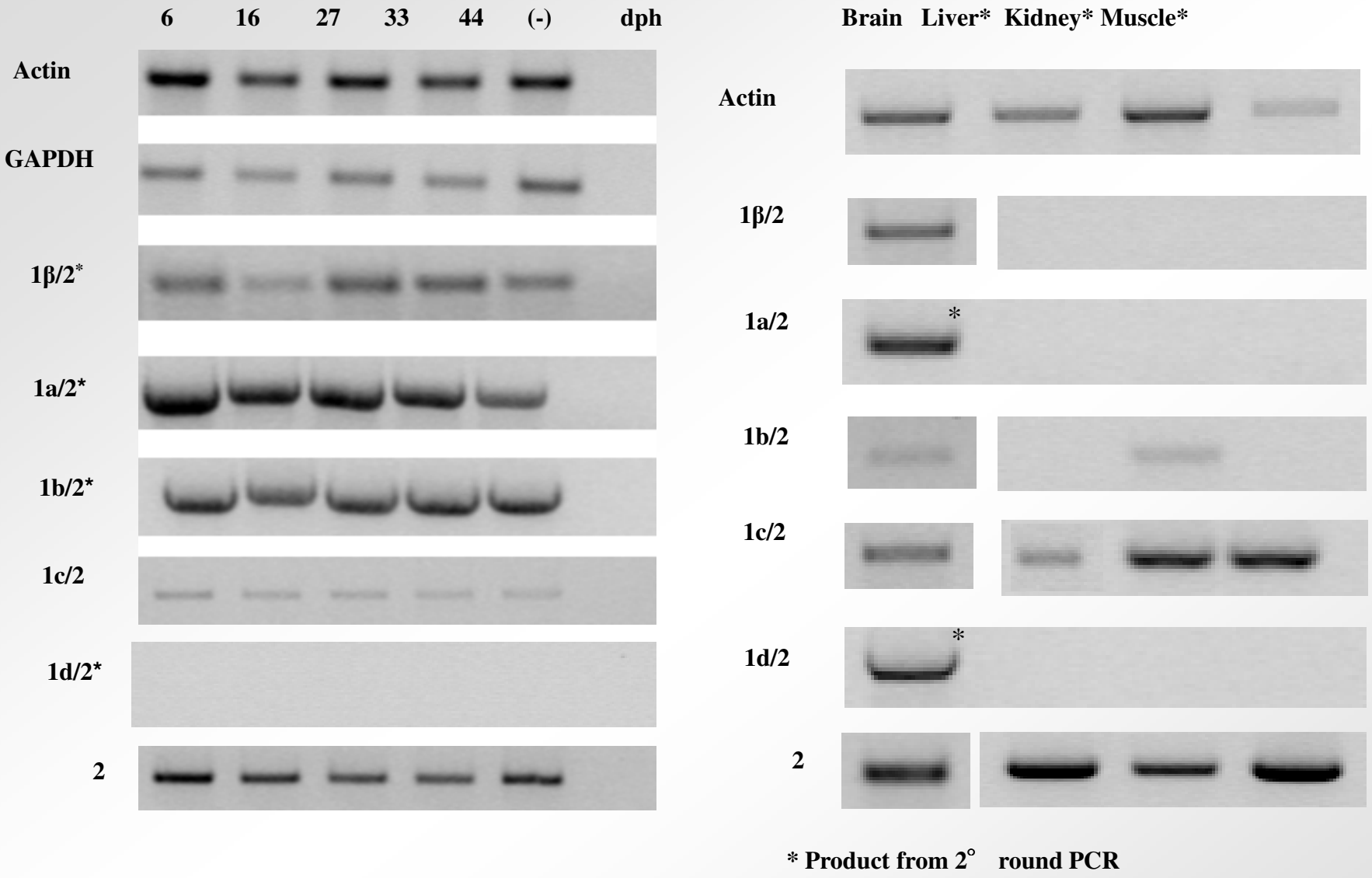
According to the most recent rat's gene structure, each of eight exons, at 5'-end, owns a promoter which regulates the transcription in mouse and rat (Aid et al., 2007).

Zebrafish BDNF has multiple promoters, too (Heinrich e Pagtakhan, 2004).

In human the regions upstream of each exon is able to activated the CAT transcription. Moreover, there is an anti-BDNF with only one promoter upstream exon I (Pruunsild et al., 2007).



There is also a negative regulation due to DNA methylation and histone deacetylation



Example of expression of Dicentrarchus labrax alternative mRNAs during larva development (left panel) and in adult tissues (right panel) obtained by RT-PCR.
*: Aliquots of first PCR products were amplified in 2nd round of PCR.

PROTEIN SEQUENCE IN *D. labrax*

MTILFVTMVISYFSCMRAAPLRDAPGMRGHRTEGYLGAAATAARGHGTPQSGGGPGQRG
ELPSLTDTFEQVIEELLEVEGEAAQLGQGADKSQGGGGPSSVVTTEAKDVDLYDSRVMI
SNQVPLEPPLLFLLEEYKNYLDAANMCMRVRRHSDPSRRGELSVCDSISQWVTAVDKKT
AIDMSGQTVTVMEKVPVPNGQLKQYFYETKCNPMGYTKEGCRGIDKRHYNSQCRTTQSY
VRALTMDSKKKIGWRFIRIDTSCVCTLTIKRGR

Signal peptide

Pro BDNF (32 kDa)

Mature BDNF (14 kDa)

3D STRUCTURE

Robinson et al., 1995

Eterodimer
BDNF/NT3

WHAT DOES PROTEIN INTERACT WITH?

BDNF dimer ⟺ TrkB

(mature protein)

TrkB dimerization and autophosphorilation; activation of different signal pathways in the cell.

- Cellular survival is helped by activation of the PI-3-kinase and the MAP-kinases (expression of antiapoptotic proteins)

- Long Term Potentiation

BDNF and TrkB are involved in the neuroendocrine control of energy balance and feeding behaviour

Xu et al., 2003

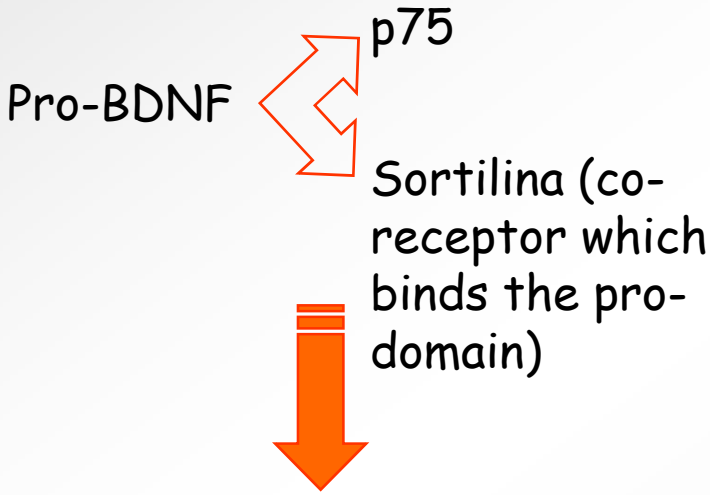
- Food deprivation reduces the level of BDNF mRNA in the hypotalamus
- Genetic mutations which determine a deficiency in BDNF or TrkB production cause obesity and hyperphagia
- TrkB acts downstream of the melanocortin-4 receptor, a protein implied in a pathway of hypotalamic regulation which controls appetite using hormones such as leptin and insulin (↓Mc4r-↓BDNF)

Tsao et al., 2008

- TrkB is a terapeutic target to cure obesity in mice

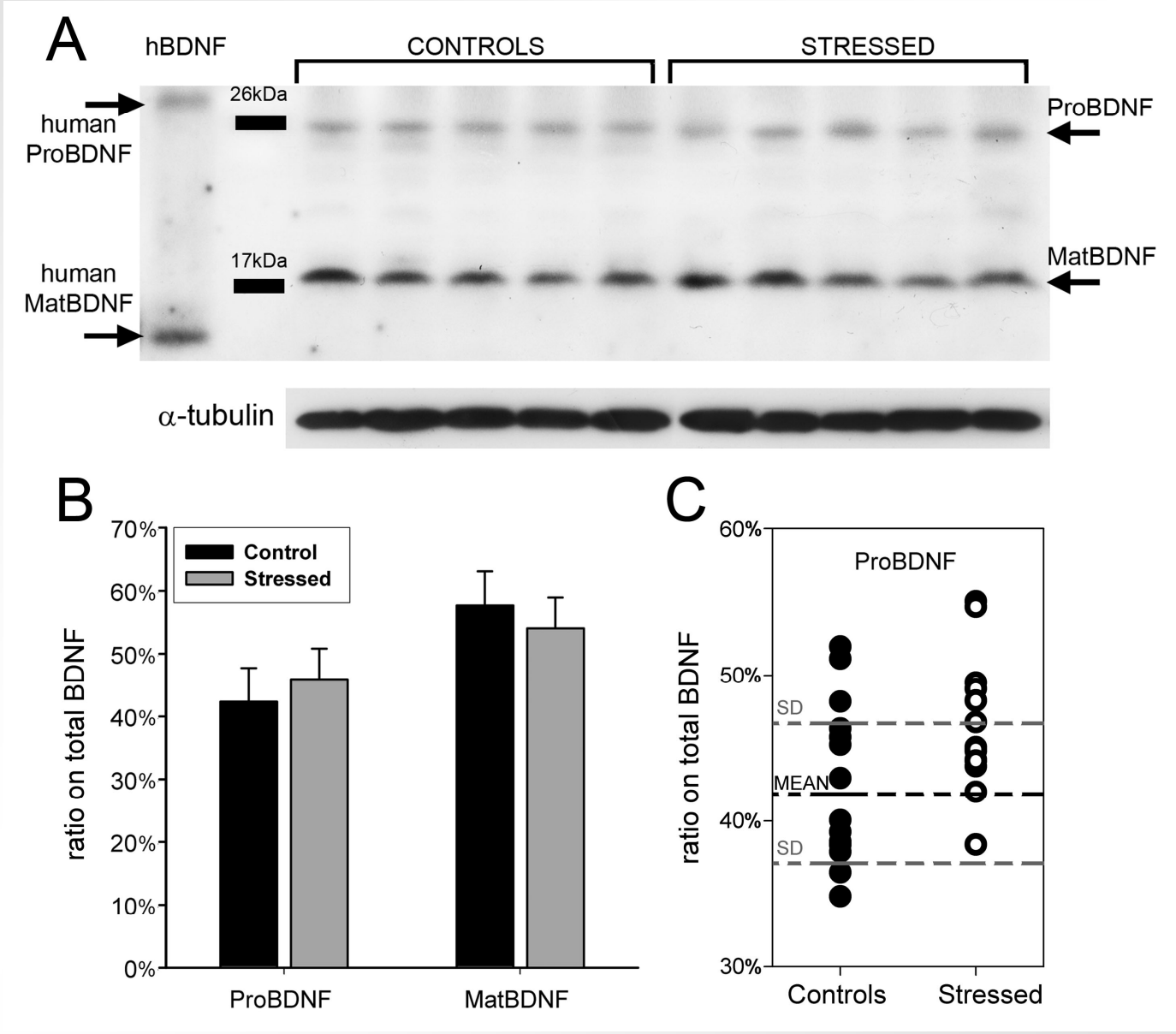
Lin et al., 2008

- Experiments in primates have shown opposite effects (anorexigenic vs orexigenic) of TrkB agonist accoriding to the kind of administration (central vs peripheral)

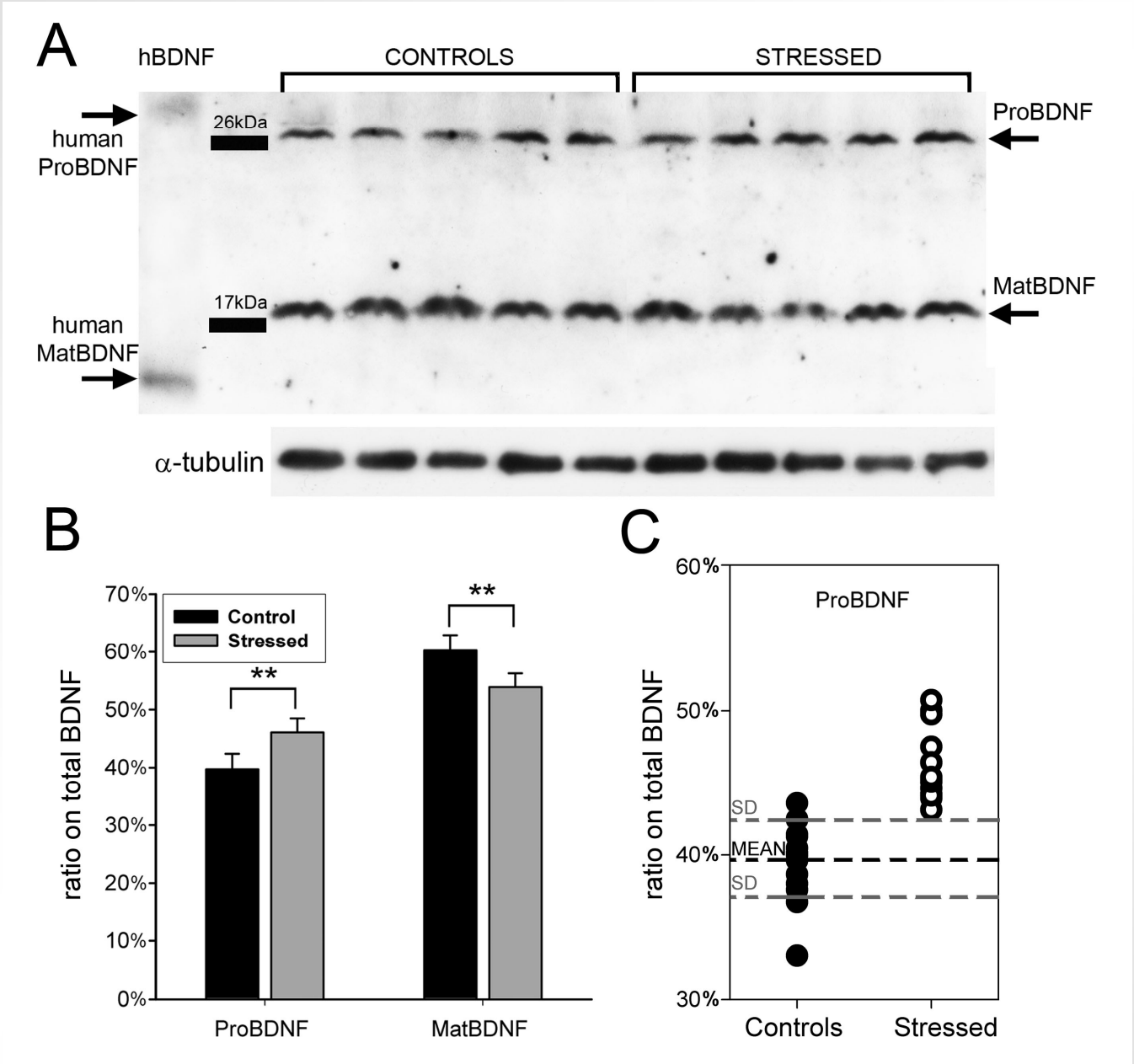


- Apoptosis (elimination of damaged cells)
- Long Term Depression

LIVER


A
hBDNF
CONTROLS
STRESSED
26kDa
human
ProBDNF
ProBDNF
17kDa
human
MatBDNF
MatBDNF
α-tubulin
B
70%
60%
50%
40%
30%
20%
10%
0%
Control
Stressed
ratio on total BDNF
ProBDNF
MatBDNF
C
60%
50%
40%
30%
ProBDNF
SD
MEAN
SD
ratio on total BDNF
Controls
Stressed

BRAIN


A
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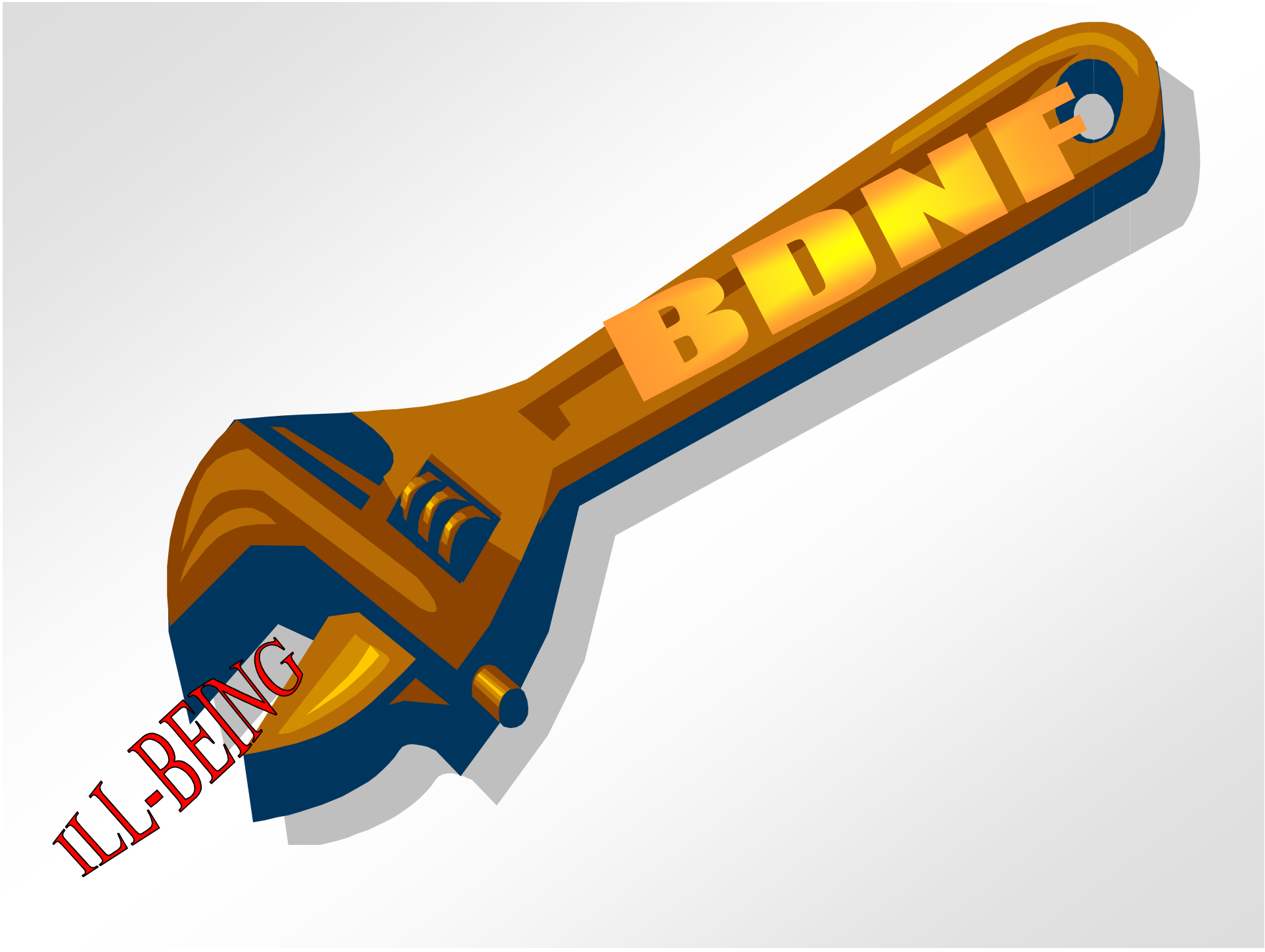
B
70%
60%
50%
40%
30%
20%
10%
0%
ratio on total BDNF
Control
Stressed
**
**
ProBDNF
MatBDNF
C
60%
50%
40%
30%
ratio on total BDNF
ProBDNF
SD
MEAN
SD
Controls
Stressed


BDNF
ILL-BEING