FINAL REPORT Grant Agreement PIEF-GA-2011-303197 Obesity and Light

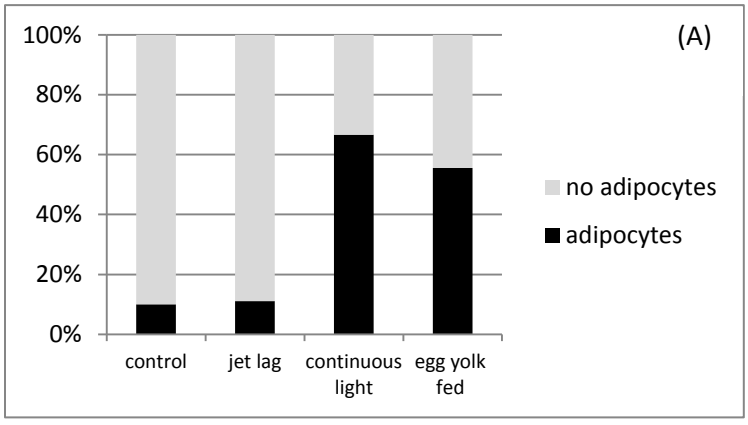
PROGRESS TOWARDS OBJECTIVES, SIGNIFICANT RESULTS, DEVIATIONS, REASONS FOR FAILING, CORRECTIVE ACTIONS

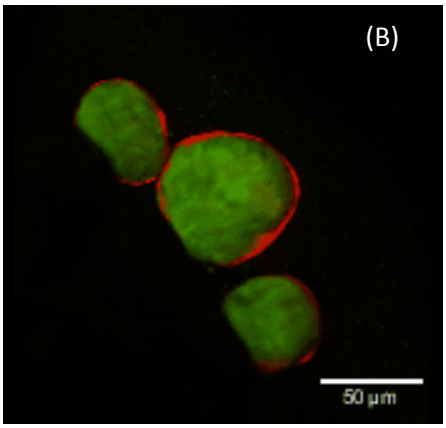
For the total period (2 years) three main objectives were proposed:

1.) How do light-induced alterations in circadian rhythmicity influence lipid metabolism in zebrafish?

The influence of variable light/dark cycles on the physiology of zebrafish should be monitored *in vivo*, under conditions in which activity and food uptake are registered. Lipid metabolism should be quantified with both *in vivo* lipid staining and measurement of various biochemical parameters including total fatty acids, triglycerides, cholesterol and hormones. The results should be supported by mRNA expression quantification of clock- and metabolism-relevant genes. This objective should answer the question whether alterations in light/dark cycles in zebrafish cause augmented lipid storage as a direct consequence of circadian and metabolic pathways or by changes in activity or food uptake.

To fulfill this objective we exposed zebrafish larvae to control light/dark conditions (14 h light and 10 h dark), altered light/ dark conditions (alternating prolonged and shortened light periods; jet lag protocol) or continuous light. Feeding was strictly controlled regarding timing and amount. Furthermore, to compare the results to diet induced obesity we fed zebrafish larvae with a hypercaloric diet, boiled chicken egg yolk. Activity was monitored using the ViewPoint ZebraBox System and adipocytes were visualized by applying CARS microscopy (collaboration with MPI Mainz, Germany) a labeling-free method which displays structures by their characteristic intrinsicvibrational contrast. Furthermore, RNA samples were taken and mRNA expression of 14 genes measured. An 7-fold increase in the appearance of adipocytes was seen in the continuous light group but no induction appeared in the jet lag group. mRNA expression of all clock genes and particularly rev-erbα were affected. Also pparβδ was affected by changes in circadian rhythms as well as hypercaloric feeding. A significant linear correlation between rev-erbα and pparβ mRNA expression was seen in zebrafish larvae exposed to continuous light. Furthermore, monitoring activity showed a reduced total activity in jet lag larvae but no difference in continuous light exposed larvae, demonstrating that the observed increase in adipogenesis was not due to less energy consumption.





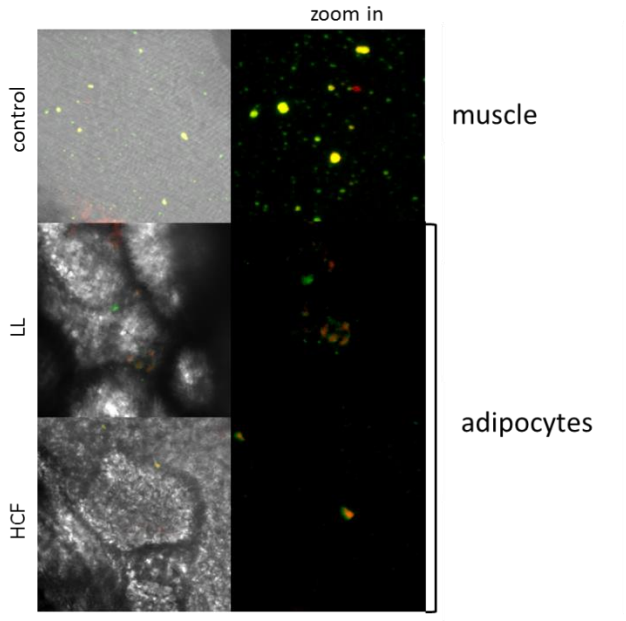
Percentage of 15 day old zebrafish larvae which developed adipocytes after 7 days of exposure (A) and adipocytes visualized by CARS microscopy (B).

2.) <u>How does rev-erbα and PPARs connect circadian and metabolic signaling pathways?</u>

It is known that the clock gene rev-erbα is induced during adipogenesis but its regulatory mechanisms are not clear. A rev-erbα knockout zebrafish line will be established and examined for changes in PPAR isoform expression in order to link these signaling pathways. The re-introduction of an rev-erbα::GFPconstruct will make Rev-erbα detectable (no antibody against Rev-erbα is commercially available). The tagged Rev-erbα will allow for quantification, localization and the detection of interaction partners. Examination of lipid metabolism in this novel transgenic line with the methods described above will also further elucidate the functional role of rev-erbα in lipid metabolism.

To fulfill this objective a TALEN construct recognizing the rev-erbα gene and inducing a point mutation was built using the addgene TALEN kit 2.0. The TALEN construct was injected into one cell stage zebrafish embryos. As no phenotype was observable and multiple repeats did not show any success we stopped the attempt to make a knock-out and a transgenic zebrafish line and decided to develop an antibody against rev-erbα to get information on protein level.

Immunostaining of larvae exposed to continuous light or being fed with hypercaloric diet showed Rev-erbα as well as Pparγ protein expression in adipocytes with both proteins being co-localized. The correlated mRNA expression of *rev-erbα* and *pparβδ*, the fact that the promoter region of *pparβδ* include three binding sites for Rev-erbα as well as the co-localization of Rev-erbα and Pparγ within the adipocytes gave new insights in the linking of circadian rhythms and adipogenesis.

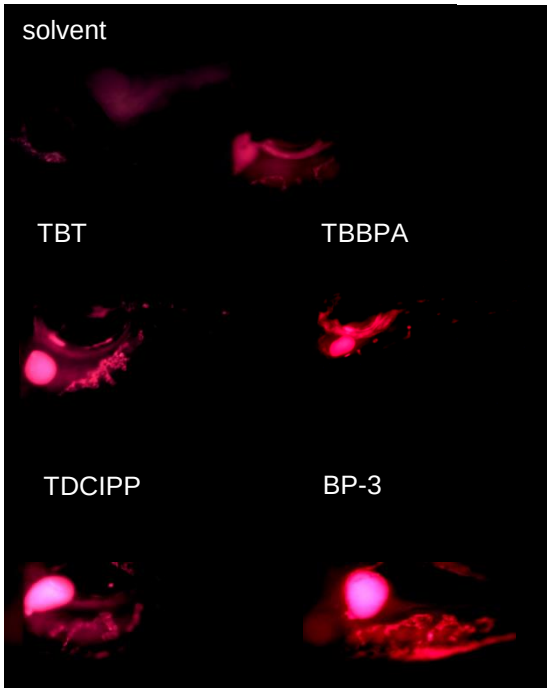


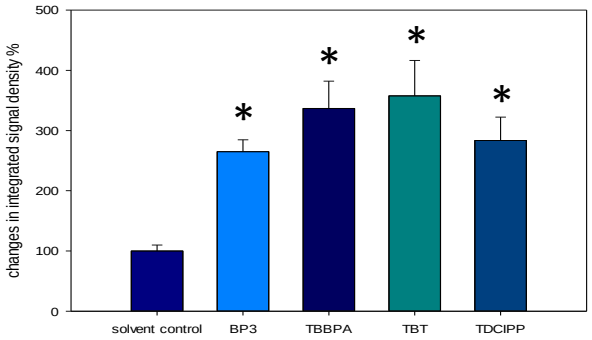
3.) Objective 3: Do obesogenic chemicals influence lipid metabolism through changes in circadian rhythm?

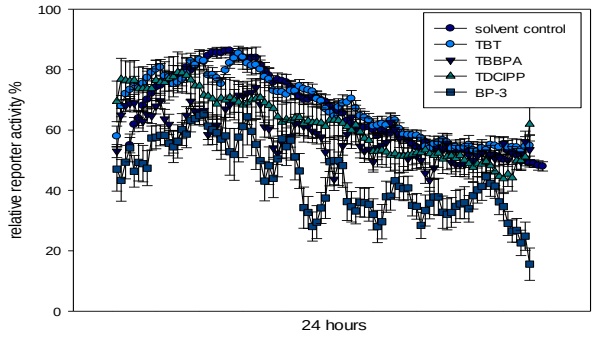
As representative obesogenic chemicals, the PPARγ agonist TBT and the PPARα agonist DEHP will be tested in zebrafish larvae at different developmental stages. These two chemicals are known obesogens in rodents, and preliminary studies in the host's laboratory have indicated the exposure of zebrafish larvae to low concentrations of TBT from 3 to 8 dpf leads to increased total fatty acids. In addition, other candidate chemicals will be tested, such as 4-azapyrene, a polycyclic aromatic compound identified in the host's laboratory to cause developmental toxicity and alter gene expression of clock genes per2 and cry1 (Hawliczek et al, 2011, submitted). Chemical treatment will be performed simultaneously with altered light/dark cycles, using the methodology developed in Objective 1, in order to mimic circadian disruption. Effects of these two stressors (light and chemicals) on lipid metabolism will be analyzed using the methods described above. In addition, clock gene and PPAR isomer expression will be measured (see Objective 1) to examine effects of chemical treatment on both lipid metabolism and circadian rhythm signaling pathways. Furthermore rev-erbα knock-out zebrafish (Objective 2) will be analyzed following chemical exposure to see whether rev-erbα plays a role in the obesogenic effects of chemicals. Internal and water concentrations of test chemicals will be analyzed using state of the art chemical analytical instrumentation available in the host's laboratory (e.g. gas and liquid chromatography). This will allow for comparison of actual exposure concentrations with environmental concentrations of test chemicals, to determine margins of safety.

Dr. Thomas Dickmeis from the KIT (Karlsruhe, Germany) gave us a transgenic zebrafish line (Tg(4xEbox:LUC) which shows reporter activity when the core clock gene CLOCK binds to a luciferase linked promoter region. We developed an exposure setup by exposing zebrafish embryos to different concentrations of environmental chemicals to determine lowest effect concentrations which we used for the experiments. We could show that environmental chemicals with obesogenic potential disturb circadian clocks and vice versa: exposure of zebrafish larvae to endocrine disrupting chemicals not only increased lipid accumulation remarkably but also affected core clock activity while exposure to known clock modulating chemicals increased lipid accumulation, even more than obeogens did. Our data clearly reveal a novel mechanism of action of environmental obesogens and strengthen the bidirectional link between circadian rhythms and lipid metabolism. This newly observed effect of environmental chemicals widens their toxic potential and therefore might change the perspective on risk assessment.

As we were not successful with making the rev-erbα knockouts (objective 2) the exposures could not be repeated to see whether rev-erbα plays a role in the obesogenic effects of chemicals.







RESEARCHER TRAINING ACTIVITIES/TRANSFER OF KNOWLEDGE/ INTEGRATION ACTIVITIES

Renate Kopp attended a course on Laboratory Animal Science at the University of Utrecht (15. – 26.04.2013), a workshop on behavior technology (ViewPoint's Zebrafish Workshop, 08.07.2013, Barcelona) and a workshop on linking endocrine disrupting chemicals and obesity (Final Workshop of the OBELIX project, 23.10.2013, Brussels). Furthermore she participated in giving a course on zebrafish embryonic toxicity tests and in supervising a Master student. She also started a collaboration with the Institute of Polymer Research at the Max Planck Institute Mainz (Germany) and the Institute for Biophototnics and Medical Imaging at the VU University Amsterdam (Netherlands). Renate Kopp attended the 8th European zebrafish meeting (Barcelona, 09.–13.07.2013) and gave a poster presentation (Zebrafish as a model for understanding the role of environmental cues in developing obesity). She also presented a poster (Bringing Obesity to Light) at the Keystone Symposium on Molecular and Cellular Biology - Obesity: A Multisystems Perspective (Vancouver, 12.-17.01.2014). To improve her knowledge about environmental toxicology she joined the SETAC meeting (Basel, 11.-15.05.2014). At the International Symposium & Workshop: Fish and amphibian embryos as alternative models in toxicology and teratology (Paris, 01-02.12.2014) she gave a poster (A transgenic zebrafish line for effect analysis of environmental compounds on circadian clocks) as well as an oral presentation (Routine Handling Affects Activity Patterns of Developing Zebrafish). Furthermore, Renate Kopp presented her data regularly within the working group and the institute.
The collected data will be published in three scientific papers which are under preparation (BRINGING OBESITY TO LIGHT: Rev-erbα, a central player in light induced adipogenesis in the zebrafish?; The Ticking of Environmental Obesogens: chemicals disturbing both lipid metabolism and circadian rhythms; Routine Handling Affects Activity Patterns of Developing Zebrafish).

STATEMENT ON THE USE OF RESOURCES, IN PARTICULAR HIGHLIGHTING AND EXPLAIING DEVIATIONS BETWEEN ACTUAL AND PLANNED RESEARCHER-MONTH IN ANNEX 1(DESCRIPTION OF WORK).

Critical goals of objective 2 have not been achieved during the first period due to technical reasons. So a new strategy has been worked out to be able to fulfill objective 2. Thus, still the goal of objective 2 could be reached.

PUBLISHABLE SUMMARY

The incidence of obesity and its associated metabolic abnormalities like diabetes and hypertension have grown to epidemic proportions worldwide (Finucane et al., 2011). Nevertheless there is still much uncertainty about underlying physiological mechanisms and the current incidence of obesity cannot be fully explained by genetic, socio-economic factors or nutrition. Increasing evidence indicates that also environmental factors contribute to the etiology of obesity, including disturbance of circadian rhythms (Gooley and Chua, 2014) and exposure to environmental chemicals (Janesick and Blumberg, 2011). Circadian rhythms, endogenously driven cycles of roughly 24 hours, time biological clocks and coordinate biochemical, physiological and behavioral processes according to the organisms' active periods. Also genes which regulate lipid synthesis and fatty acid oxidation are clock controlled (Gooley and Chua, 2014). Though disturbing circadian clocks has profound implications for human health, little is known about chemically induced alterations in clock activity and how they affect metabolic aspects like lipid accumulation and adipogenesis. The zebrafish (*Danio rerio*) is an established model for analyzing obesogenic and circadian effects. We could show that under continuous light but not under shifted light dark cycles a 7 fold higher number of zebrafish larvae developed adipocytes, even more than zebrafish larvae which were fed with hypercaloric diet. This could be due to altered expression of light driven clock genes that are linked to genes involved in adipogeneis. Adipogenesis is mainly regulated by three isoforms of the peroxisomal proliferator activated receptors (Ppars) and *pparβδ* shows several binding sites for the clock gene Rev-erbα. Thus, the regulatory pathways of circadian clocks and adipogenesis might be linked via these genes. The newly observed co-localization in zebrafish adipocytes of Rev-erbα and Pparγ, which can be activated by Pparβδ, is emphasizing a direct interplay. Furthermore, we demonstrated that environmental chemicals with obesogenic potential disturb circadian clocks and vice versa: exposure of zebrafish larvae to endocrine disrupting chemicals not only increased lipid accumulation remarkably but also affected core clock activity while exposure to known clock modulating chemicals increased lipid accumulation. Our data clearly strengthened the bidirectional link between circadian clocks and lipid metabolism and moreover revealed a novel mechanism of action of environmental obesogens. This first described effect widens the potential health implications of exposure. As the circadian clock regulates a wide variety of physiological processes, information about environmental compounds that disturb the biological clock may provide also important insights on the environmentally induced development of cancer, metabolic diseases or sleeping disorders. Furthermore, it might provide possible explanations for the increasing number of people suffering from obesity and might even offer new treatment approaches.