

# The Circadian Clock Components CRY1 and CRY2 Are Necessary to Sustain Sex Dimorphism in Mouse Liver Metabolism\*

Received for publication, November 3, 2008, and in revised form, February 2, 2009. Published, JBC Papers in Press, February 11, 2009, DOI 10.1074/jbc.M808360200

Isabelle M. Bur<sup>†1</sup>, Anne M. Cohen-Solal<sup>‡</sup>, Danielle Carmignac<sup>§</sup>, Pierre-Yves Abecassis<sup>¶</sup>, Norbert Chauvet<sup>‡</sup>, Agnès O. Martin<sup>‡</sup>, Gijsbertus T. J. van der Horst<sup>||</sup>, Iain C. A. F. Robinson<sup>§</sup>, Patrick Maurel<sup>\*\*</sup>, Patrice Mollard<sup>‡</sup>, and Xavier Bonnefont<sup>‡2</sup>

From <sup>†</sup>CNRS, UMR 5203, Institut de Génomique Fonctionnelle and INSERM, U661 and Université Montpellier, 34094 Montpellier, France, the <sup>§</sup>National Institute of Medical Research, Division of Molecular Neuroendocrinology, The Ridgeway, Mill Hill, London NW7 1AA, United Kingdom, <sup>¶</sup>Sanofi-Aventis, Discovery Metabolism and Pharmacokinetics Safety (DMPK-S), 3 Digue d'Alfortville, 94140 Alfortville, France, the <sup>||</sup>Erasmus University Medical Center, Department of Genetics, Dr. Molewaterplein 50, 3000 CA Rotterdam, The Netherlands, and <sup>\*\*</sup>INSERM, U632, 1919 Route de Mende, 34293 Montpellier, France

In mammals, males and females exhibit anatomical, hormonal, and metabolic differences. A major example of such sex dimorphism in mouse involves hepatic drug metabolism, which is also a noticeable target of circadian timekeeping. However, whether the circadian clock itself contributes to sex-biased metabolism has remained unknown, although several daily output parameters differ between sexes in a number of species, including humans. Here we show that dimorphic liver metabolism is altered when the circadian regulators *Cryptochromes*, *Cry1* and *Cry2*, are inactivated. Indeed, double mutant *Cry1*<sup>-/-</sup> *Cry2*<sup>-/-</sup> male mice that lack a functional circadian clock express a number of sex-specific liver products, including several cytochrome P450 enzymes, at levels close to those measured in females. In addition, body growth of *Cry*-deficient mice is impaired, also in a sex-biased manner, and this phenotype goes along with an altered pattern of circulating growth hormone (GH) in mutant males, specifically. It is noteworthy that hormonal injections able to mimic male GH pulses reversed the feminized gene expression profile in the liver of *Cry1*<sup>-/-</sup> *Cry2*<sup>-/-</sup> males. Altogether, our observations suggest that the 24-h clock paces the dimorphic ultradian pulsatility of GH that is responsible for sex-dependent liver activity. We thus conclude that circadian timing, sex dimorphism, and liver metabolism are finely interconnected.

Phenotypic differences between males and females of a given species exist from invertebrates to humans and cover various features including disease susceptibility and life span, for example. Although the anatomical and hormonal differences between sexes are well described, few genetic determinants are

known to account for sexual dimorphism in mammals. Recent genome-wide studies on various mouse tissues showed that major differences in gene expression between males and females involve drug metabolism (1, 2), and among somatic organs, the sex-biased transcriptional activity is particularly high in the liver (3–5). These observations may provide insight into why many drugs exhibit a faster clearance in women as compared with men (6) but also underscore the importance of considering sex issues in studies on liver metabolism.

Interestingly, liver activity also fluctuates along the light-dark cycle and is a noticeable target of circadian timekeeping (7–9). Both systemic cues and a circadian clock within hepatocytes are involved in daily oscillations of liver metabolism (10, 11). However, because most of these studies were focused on males exclusively, the possible interaction between circadian oscillators and sex dimorphism of hepatic activity is not documented.

This prompted us to investigate whether circadian cogwheels could also be involved in the sexual dimorphism observed at the hepatic level. Indeed, several daily output parameters differ between sexes in a number of species, including humans (12–14), which may reflect the ability of the suprachiasmatic nuclei (SCN),<sup>3</sup> where the central pacemaker resides, to sense sexual hormones through receptors to androgens and estrogens (15, 16). We therefore assessed liver activity in homozygous double mutant *Cryptochrome 1* and *2* males and females (hereafter referred to as *Cry*<sup>-/-</sup> mice) that are devoid of a functional circadian clock (17). Our results reveal that *Cry*<sup>-/-</sup> males have a feminized liver and pinpoint dimorphic GH pulsatility as the main signal altered by the inactivation of *Cryptochromes*.

## EXPERIMENTAL PROCEDURES

**Quantitative Real-time PCR**—Eight-week-old *Cry*<sup>-/-</sup> (17) and isogenic control mice, maintained under a 12-h light/12-h dark cycle (ZT0 and ZT12 refer to lights on and off, respectively) and free access to food and water, were sacrificed by cervical dislocation. Livers were rapidly removed, frozen in liquid nitrogen, and stored at -80 °C until use. Total RNA extraction was done with the RNeasy kit (Qiagen) according to the

\* This work was supported by the Netherlands Organization for Scientific Research Grant ZonMW Vici 918.36.619 to G. T. J. v. d. H., a Marie Curie European Reintegration Grant from the European Community (to X. B.), a grant from the Région Languedoc-Roussillon (to P. M.), a grant from the Institut National de la Santé et de la Recherche Médicale (to P. M. and X. B.), and a grant from the Centre National de la Recherche Scientifique (to P. M. and X. B.).

<sup>1</sup> Fellow of the Ministère de l'Enseignement Supérieur et de la Recherche.

<sup>2</sup> To whom correspondence should be addressed: Institut de Génomique Fonctionnelle, 141 Rue de la Cardonille, 34094 Montpellier Cedex 5, France. Tel.: 33-467142989; Fax: 33-467542432; E-mail: Xavier.Bonnefont@igf.cnrs.fr.

<sup>3</sup> The abbreviations used are: SCN, suprachiasmatic nuclei; *Cry*, Cryptochrome; Cyp, cytochrome P450; GH, growth hormone; OHT, hydroxylated testosterone; MUP, major urinary protein; STAT5b, signal transducer and activator of transcription 5b; ZT, zeitgeber time; ANOVA, analysis of variance.

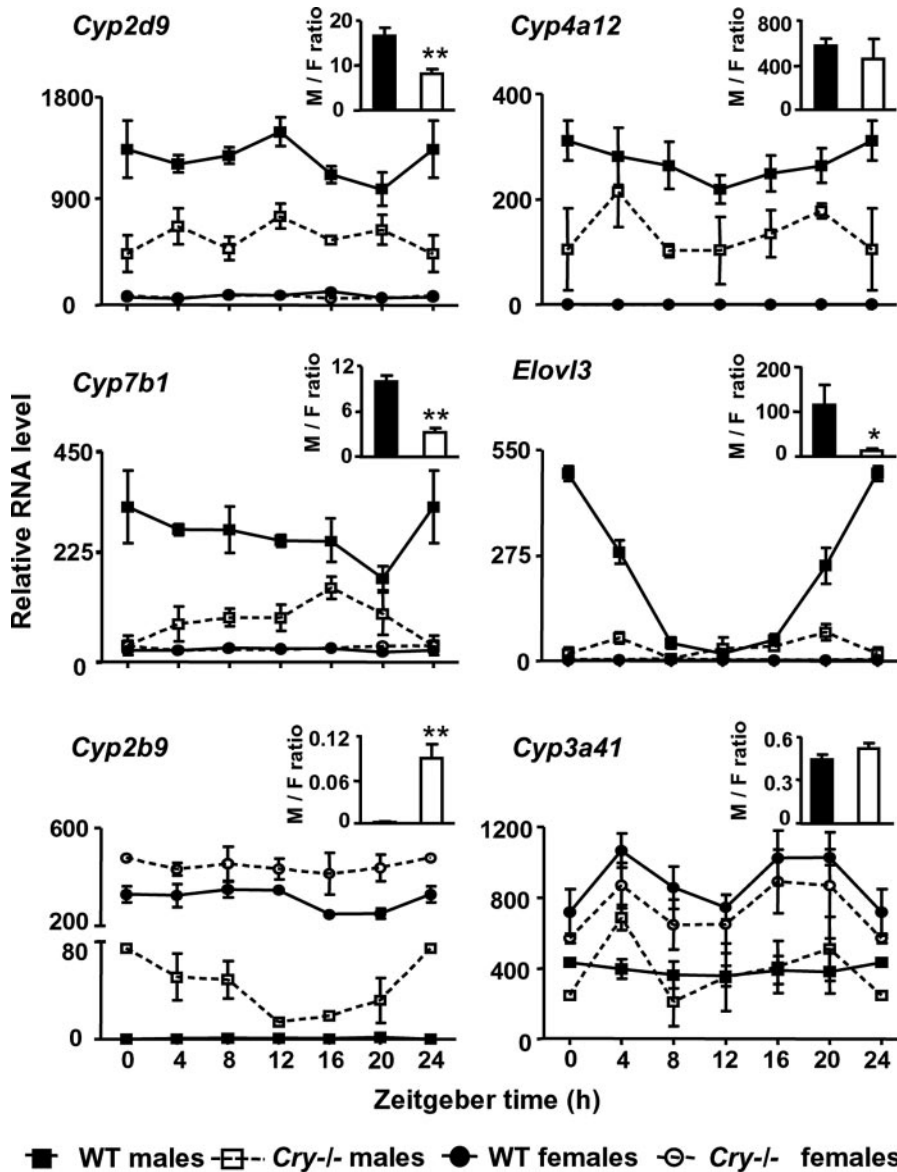


FIGURE 1. The sex dimorphism in gene expression is reduced in the liver of *Cry*<sup>-/-</sup> mice. Livers from control and *Cry*<sup>-/-</sup> mice were collected every 4 h. Relative RNA levels, measured by quantitative PCR, are shown for control wild type (WT, closed symbols, solid lines) and *Cry*<sup>-/-</sup> (open symbols, dotted lines) males (squares) and females (circles), respectively. Data from ZT0 are replotted at ZT24 to improve readability. RNA levels are graphed as means  $\pm$  S.E.,  $n = 3-4$  for each series. For all genes, the global statistical significance is  $p < 0.005$  between control males and females and between males of both genotypes (two-way ANOVA). Insets show the average ratio of expression over 24 h between male and female livers (M/F ratio) in wild type (solid bars) and *Cry*<sup>-/-</sup> mice (open bars). \*\*,  $p < 0.01$  and \*,  $p < 0.05$  as compared with control males, Mann-Whitney U test.

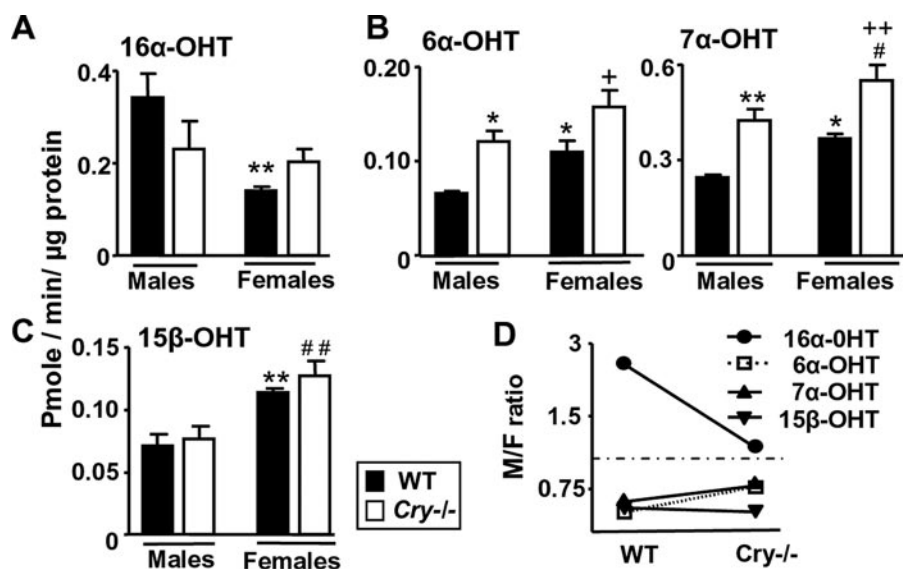
manufacturer's instructions. Total RNA was then reverse-transcribed into cDNA by using Superscript III reverse transcriptase (Invitrogen) with oligonucleotide random hexamers. Primers of the mouse mRNAs to be investigated were designed using Primer Express (Applied Biosystems) or Beacon Designer (Premier Biosoft) and checked for specificity using the BLAST program. In general, PCR primers were chosen to introduce a minimum of a two-nucleotide mismatch with all related *Cyp* subfamily members. Results presented for *Cyp3a41* may actually represent the combined contribution of *Cyp3a41* and *Cyp3a16* RNA because the primers bring only one single nucleotide mismatch in between the two forms and theoretical amplicons with 96% homology that may not be distinguished by

the dissociation curve of amplified products conducted to verify the unicity of amplified products. Real-time PCR was performed and analyzed using a 7500 Fast real-time PCR system with SYBR Green master mix (Applied Biosystems). Relative expression levels were determined using the comparative  $C_T$  method to normalize target gene mRNA to *Gapdh*.

**Microsome Preparation and Assessment of Cytochrome P450 Enzymatic Activity**—Two-month-old mice were sacrificed at ZT4, and their livers were rapidly dissected, homogenized in homogenizing buffer (10 mM Tris-HCl, pH 7.4, 1 mM EDTA, and 250 mM sucrose) with the addition of phosphatase inhibitors (1 mM sodium orthovanadate and 10 mM sodium fluoride) and a protease inhibitor (100  $\mu$ M phenylmethylsulfonyl fluoride) and centrifuged at 9000 rpm for 15 min to obtain a total tissue homogenate. Microsomal pellets were separated from cytosolic supernatant by ultracentrifugation and suspended in potassium phosphate buffer, pH 7.4, containing 0.1 mM EDTA and 20% glycerol. Microsomal protein concentrations were determined using the Bradford assay.

Testosterone hydroxylase activity was measured as described previously (18). Liver microsomes were tested at 1 mg of proteins/ml in 0.05 M Tris buffer, pH 7.4, for testosterone hydroxylase activity using 300  $\mu$ M of <sup>14</sup>C-labeled testosterone as a substrate. In addition, 5 $\alpha$ -reductase activity was inhibited by the addition of 5  $\mu$ M finasteride to the incubation mixture. The NADPH-regenerating system was composed of 10 mM glucose-6-phosphate, 5 IU/ml glucose-6-phosphate dehydrogenase, and 10 mM MgCl<sub>2</sub> in 0.05 M Tris buffer, pH 7.4. The reaction was started by the addition of 1 mM NADPH. The incubation, at 37  $^{\circ}$ C, lasted 15 min, and the reaction was stopped using a mixture of 3 ml of dichloromethane and 65 nmol of 11 $\beta$ -hydroxytestosterone (UV internal standard). Each sample was vigorously vortexed and centrifuged. Two ml of organic phase were transferred to a clean glass test tube and evaporated to dryness under a stream of nitrogen gas. Nitrogen-dried dichloromethane extracts were dissolved in a mixture of 0.1 M ammonium acetate/methanol/acetonitrile. Testosterone metabolites were separated by reverse phase high pressure liquid chroma-

## Sex Dimorphism in the Liver Depends on Circadian Timekeeping



**FIGURE 2. Sex differences in liver microsomal testosterone hydroxylase activities are reduced in *Cry*<sup>-/-</sup> mice.** Testosterone hydroxylase activities were determined in liver microsomes prepared from wild type (WT, solid bars) and *Cry*<sup>-/-</sup> mice (open bars). Specific activities are shown for male-predominant 16 $\alpha$ -hydroxylase (A), female-predominant 6 $\alpha$ -hydroxylase and 7 $\alpha$ -hydroxylase (B), and 15 $\beta$ -hydroxylase (C). D, activity ratios between male and female microsomes are plotted on a log scale. Note that this ratio tends to 1 (horizontal dashed line) for microsomal activities measured from *Cry*<sup>-/-</sup> mice in A and B, whereas it remains constant for 15 $\beta$ -hydroxylase activity. \*\*,  $p < 0.01$  and \*,  $p < 0.05$  as compared with control males, ++,  $p < 0.01$  and +,  $p < 0.05$  as compared with control females and ##,  $p < 0.01$  and #,  $p < 0.05$  as compared with *Cry*<sup>-/-</sup> males; symbols are omitted when differences are not significant (two-way ANOVA;  $n = 5$  animals in each condition, means  $\pm$  S.E.).

tography, using Symmetry C18 column 5  $\mu$ m (220  $\times$  2.1 mm), heated at 55  $^{\circ}$ C. The mobile phases used were (phase A) 0.1 M ammonium acetate; 30% methanol and (phase B) 0.1 M ammonium acetate; 70% methanol. Hydroxylated metabolites were monitored by UV at 247 nm and by radioactivity detection and eluted with a linear gradient: 0.7% of phase B/min at a flow rate of 350  $\mu$ l/min. Hydroxylated testosterone (OHT) standards (2 $\alpha$ -OHT, 2 $\beta$ -OHT, 6 $\alpha$ -OHT, 6 $\beta$ -OHT, 7 $\alpha$ -OHT, 11 $\beta$ -OHT, 15 $\alpha$ -OHT, 15 $\beta$ -OHT, 16 $\alpha$ -OHT, 16 $\beta$ -OHT) were used to identify testosterone metabolites.

**Determination of MUPs Levels in Urine**—Urine was collected at ZT4 and briefly centrifuged (3 min at 8800  $\times$  g). The supernatant was diluted (1/20) with 2 $\times$  Laemmli buffer and heated at 90  $^{\circ}$ C for 90 s. Routinely, 8  $\mu$ l of urine solution were analyzed by electrophoresis on a 15% SDS-polyacrylamide gel stained with Coomassie Blue. Relative amounts of proteins in the molecular weight range of 20,000 were estimated by integrating band density, expressed in arbitrary units.

**Hormonal Assays**—Circulating hormones were measured from trunk blood collected in heparinized centrifuge tubes for plasma preparation. For pituitary content, anterior pituitary glands were homogenized in phosphate-buffered saline. Assays were performed using a testosterone enzyme-linked immunosorbent assay kit (Cayman Chemical), according to the manufacturer's recommendations, and a GH-specific radioimmunoassay using reagents provided by A. F. Parlow.

## RESULTS

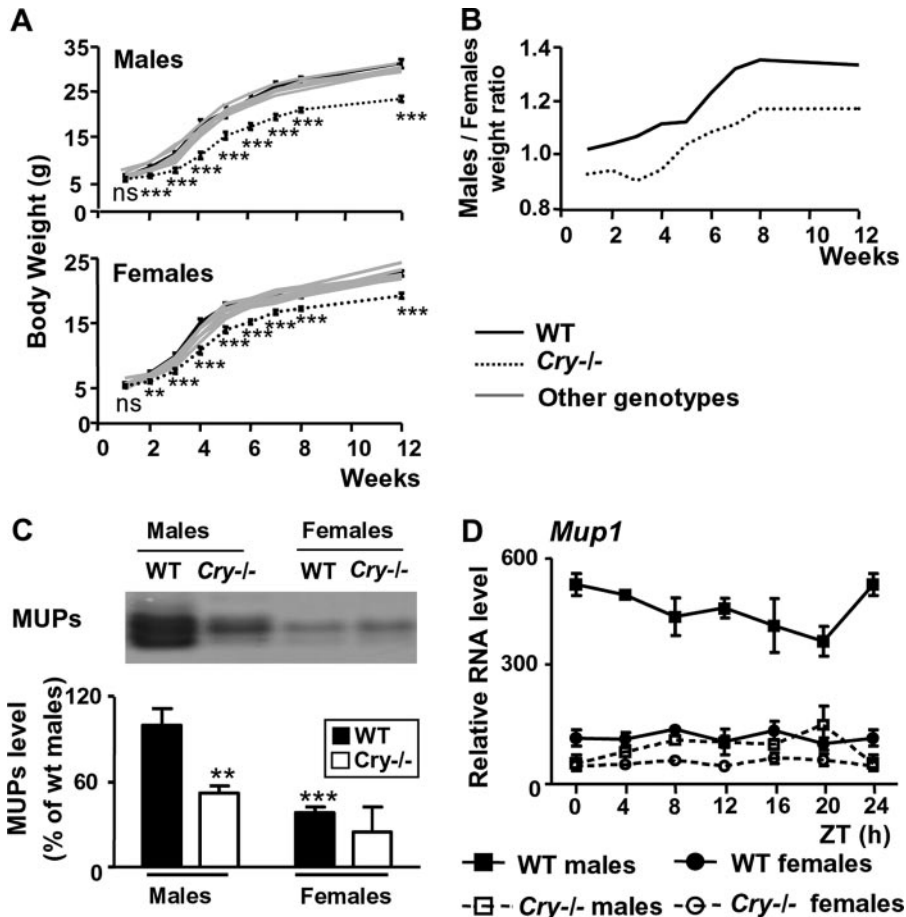
**The Sex Dimorphism in Liver Activity Is Impaired in *Cry*<sup>-/-</sup> Mice**—To determine whether circadian clock components impact on differences between males and females, we assessed by real-time quantitative PCR the expression of several dimor-

phic genes in the liver of male and female mice sacrificed at selected time points around the light-dark cycle and compared their expression level in control and double homozygous *Cry*<sup>-/-</sup> mice. Sexually dimorphic genes include members of the cytochrome P450 (*Cyp*) family that are involved in the hydroxylation of steroid hormones and various chemicals, and *Elovl3*, involved in fatty acid biosynthesis (1). In control animals, we found that the difference in expression between males and females persists all along the light-dark cycle for most of the genes considered (Fig. 1). As reported previously (19, 20), *Elovl3* exhibits daily variations in male liver and is hardly detectable in females at any time point (Fig. 1).

Interestingly, the male-female dimorphism is strongly reduced in *Cry*<sup>-/-</sup> mice. Indeed, the male-predominant genes *Cyp2d9*, *Cyp4a12*, and *Cyp7b1* are significantly down-regulated along the light-dark cycle in male *Cry*<sup>-/-</sup> livers, whereas modest, if any, alteration is noticed in females (Fig. 1). The expression of *Elovl3* becomes constitutively low, and daily variations are abolished in male livers. Conversely, the female-predominant gene *Cyp2b9* is significantly up-regulated in *Cry*<sup>-/-</sup> mutant males, which dramatically increased the expression ratio between males and females (Fig. 1, inset). Hence, inactivation of *Cry1* and *Cry2* genes leads to a feminization of the expression patterns of several genes in the male liver.

The different expression patterns of *Cyp* genes between sexes are believed to affect the rate of metabolism for a given substrate directly. To assess whether the feminized gene expression profile of *Cry*<sup>-/-</sup> males is translated at the metabolic level, we measured the activity of microsomal hydroxylases. Levels of the male-predominant testosterone 16 $\alpha$ -hydroxylase activity tend to become similar in microsomes from *Cry*<sup>-/-</sup> males and females (Fig. 2, A and D). In parallel, the sexual dimorphism in production of the female-predominant metabolites 6 $\alpha$ -OH and 7 $\alpha$ -OH testosterone is reduced in *Cry*<sup>-/-</sup> mice as compared with wild types (Fig. 2, B and D). Although testosterone metabolites cannot be reliably ascribed to individual *Cyp* forms, these findings mirror our results obtained with quantitative PCR and support that metabolic sex dimorphism is altered in *Cry*<sup>-/-</sup> mice.

It is worth noting that the feminization of *Cry*<sup>-/-</sup> males is incomplete and does not extend to all sexually dimorphic genes of liver metabolism. For example, the male-female difference in expression of the female-predominant gene *Cyp3a41* remains unchanged in *Cry*<sup>-/-</sup> animals (Fig. 1). Similarly, the female-predominant testosterone 15 $\beta$ -hydroxylase activity is not altered in either *Cry*<sup>-/-</sup> males or females (Fig. 2, C and D).



**FIGURE 3. The GH-dependent sex dimorphism is suppressed in *Cry*<sup>-/-</sup> mice.** *A*, body weight of animals obtained from *Cry1*<sup>+/-</sup>/*Cry2*<sup>+/-</sup> intercrosses. Wild type (black solid line), *Cry*<sup>-/-</sup> (dotted line) and littermates bearing at least one wild type *Cry* allele (gray solid lines), males (upper panel) and females (lower panel), were weighed weekly from 1 to 8 weeks of age and at 12 weeks. Data are graphed as means  $\pm$  S.E. \*\*\*  $p < 0.005$ , \*\*  $p < 0.01$ , and ns, not significant, for control versus *Cry*<sup>-/-</sup> mice, Mann-Whitney *U* test. *B*, male/female body weight ratios for wild type (WT, solid line) and *Cry*<sup>-/-</sup> (dotted line) mice. Note that ratio values are close to 1 in *Cry*<sup>-/-</sup> mice, indicating a loss of sexually dimorphic growth rates. *C*, MUPs accumulation. A representative example of MUPs stained with Coomassie Blue after SDS-polyacrylamide gel electrophoresis (upper panel) and pooled analysis of MUPs content in urine of wild type (solid bars,  $n = 9$  males and  $n = 14$  females) and *Cry*<sup>-/-</sup> (open bars,  $n = 15$  males and  $n = 12$  females) is shown. \*\*\*  $p < 0.005$  and \*\*  $p < 0.01$  as compared with wild type males (two-way ANOVA). *D*, *Mup1* expression level in the liver of mice used in Fig. 1. The global statistical significance by two-way ANOVA is  $p < 0.001$  between control males and females and between males of both genotypes.

Moreover, *Cry*<sup>-/-</sup> males are fertile<sup>4</sup> and exhibit normal levels of circulating testosterone ( $3784 \pm 1099$  pg/ml,  $n = 8$ , and  $2996 \pm 860$  pg/ml,  $n = 6$ , in wild type and *Cry*<sup>-/-</sup> males, respectively,  $p > 0.05$ , Mann-Whitney *U* test), which is in contrast with another mutant mouse line lacking the circadian clock protein BMAL1 (21). These observations suggest that the feminization of the liver of *Cry*<sup>-/-</sup> mutant males is not driven by impaired gonadal function. Instead, a definite pathway must be altered downstream the testicular activity, leading to the partial feminization of the liver.

**Dimorphic Traits Dependent on Ultradian GH Pulsatility Are Feminized in *Cry*<sup>-/-</sup> Males**—Ultradian profiles of circulating GH are a key mediator for sex-dependent effects on the liver in many species (3, 22–24). Indeed, a major difference between males and females in both rats and mice is the sustained (3–4 h) interpulse interval of low GH between peaks of secretion (25,

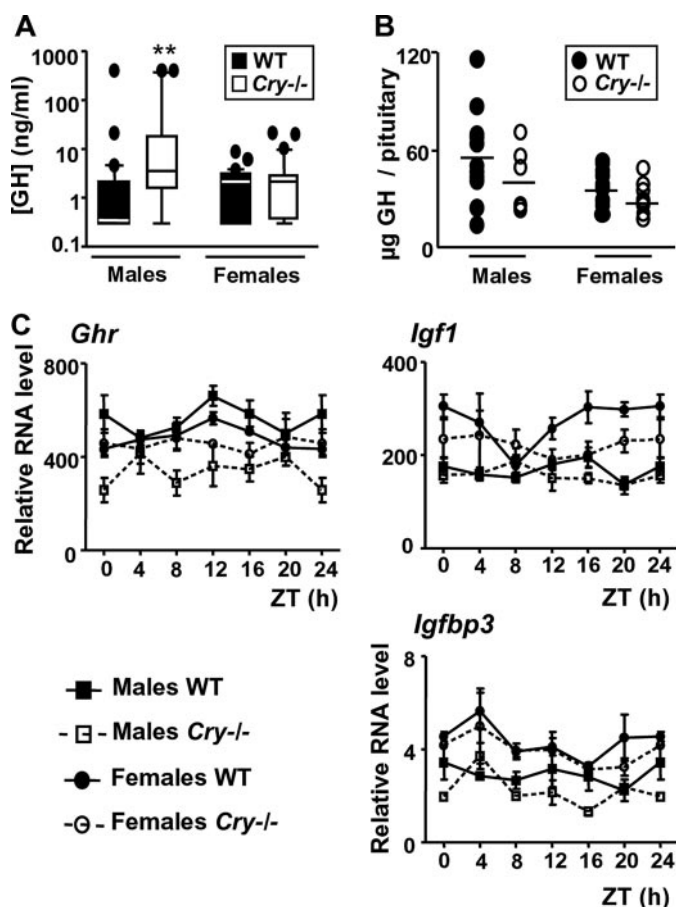
26), required for the expression of male-specific liver genes (27). These sex differences in GH patterns largely contribute to the sexual dimorphism in body growth (28). The pulsatile GH release is also well known for increasing the synthesis of major urinary proteins (MUPs) in the liver of males and their subsequent accumulation in urine (29, 30). We therefore decided to rely on these two parameters, body growth and MUPs content, as indirect readouts of GH pulsatility.

Firstly, previous studies reported that clock-deficient *Bmal1*<sup>-/-</sup> or *Cry*<sup>-/-</sup> mice are significantly smaller than control animals (31–33). This prompted us to systematically compare the postnatal growth of *Cry*<sup>-/-</sup> males and females. Double mutant *Cry1*<sup>-/-</sup> *Cry2*<sup>-/-</sup> animals of either sex exhibit a marked reduction in size and show a 10–20% reduction of body weight as compared with littermates bearing at least one wild type allele of either *Cry* gene (Fig. 3A). This growth deficit first becomes apparent at 2–3 weeks after birth, which matches the onset of the somatotroph axis (28). Importantly, the growth deficiency of *Cry*<sup>-/-</sup> mice is more pronounced in males than in females, leading to a decrease of sex dimorphism in body growth as emphasized by the lower body weight ratio between males and females in mutant animals as compared with wild type mice (Fig. 3B).

Secondly, to estimate the accumulation of MUPs, urine from matched control and *Cry*<sup>-/-</sup> males and females was collected and analyzed by electrophoresis. We found that MUPs levels are dramatically decreased in the urine of male *Cry*<sup>-/-</sup> mice as compared with wild type males (Fig. 3C). This is not due to a deficiency in urine concentration because urine osmolarity is even slightly higher in *Cry*<sup>-/-</sup> mice than in control animals ( $2397 \pm 184$  mosM/kg H<sub>2</sub>O,  $n = 12$  and  $2451 \pm 231$  mosM/kg H<sub>2</sub>O,  $n = 9$  for mutant males and females, respectively;  $1871 \pm 130$  mosM/kg H<sub>2</sub>O,  $n = 11$  and  $1873 \pm 316$  mosM/kg H<sub>2</sub>O,  $n = 7$  for wild type males and females, respectively). Rather, the decreased MUPs content in the urine of *Cry*<sup>-/-</sup> males is associated with the downregulation of the *Mup1* gene expression in their liver (Fig. 3D). Thus, both body growth and MUPs accumulation show impaired sex dimorphism in *Cry*<sup>-/-</sup> mice, suggesting that altered GH profiles may be responsible for their feminized metabolic pattern.

<sup>4</sup> I. M. Bur, A. M. Cohen-Solal, and X Bonnefont, unpublished observations.

## Sex Dimorphism in the Liver Depends on Circadian Timekeeping



**FIGURE 4. Circulating GH levels are altered in *Cry*<sup>-/-</sup> males.** *A*, box plots of circulating GH levels measured in trunk blood randomly collected between ZT2 and ZT8 from wild type (WT, solid boxes with median in white,  $n = 30$  males and 30 females) and *Cry*<sup>-/-</sup> mice (open boxes with median in black,  $n = 20$  males and 30 females) of each sex. Individual values outside 90% of the population distributions are plotted as black circles. The median values of the four groups are significantly different ( $p < 0.05$ , Kruskal-Wallis test). \*\*,  $p < 0.01$  between wild type and *Cry*<sup>-/-</sup> males (Dunn's multiple comparison test). *B*, the total amount of GH was quantified from whole pituitary glands of *Cry*<sup>-/-</sup> (open circles) and control (solid circles) males and females. The inactivation of *Cry* genes has no effect on average GH levels ( $p = 0.20$  and  $p = 0.15$  for control versus *Cry*<sup>-/-</sup> mice in males and females, respectively, Mann-Whitney *U* test). *C*, expression levels of genes involved in GH signaling were measured in the liver from mice used in Fig. 1. RNA levels are graphed as means  $\pm$  S.E.,  $n = 3-4$  for each series. The global statistical significance by two-way ANOVA is  $p < 0.001$  and  $p < 0.05$  between males of both genotypes for *Ghr* and *Igfbp3*, respectively. No significant alteration was found for *Igf1* in *Cry*<sup>-/-</sup> males as compared with controls. *Ghr*, GH receptor.

***Cry*<sup>-/-</sup> Males Exhibit Altered Circulating GH Levels**—The direct assessment of temporal GH patterns in the mouse, unlike in rat, is hampered by technical difficulties of repeated blood sampling in freely moving animals. To evaluate whether the inactivation of the *Cry* genes is associated with an altered GH secretion pattern, we collected trunk blood at random times from control and *Cry*<sup>-/-</sup> males and females. As reported previously (29), control males and females show a similar distribution of their GH levels, except that maximal values are higher in males (Fig. 4A). Interestingly, 53% of the values obtained from male blood (16/30) were below 1 ng/ml, whereas this proportion falls to only 23% in females (7/30). It is likely that such a difference is the footprint of the long and short durations of GH troughs in between secretion peaks in

males and females, respectively, as described in the single study that reported direct measurements of GH pulsatility in the mouse (25). The *Cry* deletion did not significantly alter the distribution of GH values in females. In contrast, GH levels were significantly shifted higher in *Cry*<sup>-/-</sup> males (Fig. 4A). Only 20% of random GH values were below 1 ng/ml (4/20), suggesting that GH trough duration is shorter than in control males. These data suggest that the ultradian GH profile is altered in *Cry*<sup>-/-</sup> males with reduced non-secreting episodes.

Accordingly, we found that total pituitary GH contents from wild type males are scattered along a wide range of values (Fig. 4B), which possibly depicts the alternating course of GH surges and troughs, whereas the narrow pattern obtained with glands from *Cry*<sup>-/-</sup> males resembles the distribution of values from females (Fig. 4B). However, although GH patterns are altered in *Cry*<sup>-/-</sup> males, their pituitary gland remains able to secrete a high amount of GH when stimulated with the intravenous injection of 100 ng of GH-releasing hormone or synthetic GH-releasing hexapeptide GHRP6 (data not shown). Moreover, although we noticed a slight down-regulation of the GH receptor, *Ghr*, in the liver of *Cry*<sup>-/-</sup> males, no important alteration were found in the somatotroph liver outputs, such as genes coding for the insulin-like growth factor *Igf1* and its binding protein *Igfbp3* (Fig. 4C).

Taken together, our data show that the *Cry*<sup>-/-</sup> mutation alters the GH tempo. Interestingly, it is already known that the internal circadian clock gets less robust (34) and that the somatotroph axis dampens (35) during aging. To investigate whether these alterations may affect sex dimorphism in liver metabolism, we compared liver gene expression between young and old male mice. We found that aged wild type male mice have a feminized liver gene pattern (Fig. 5), as reported previously in rats (27). Although slight differences can be noted, the liver of young *Cry*<sup>-/-</sup> and old wild type males are therefore submitted to similar changes as compared with young control animals (Figs. 1 and 5). Hence, we reasoned that circadian timekeeping may pace the GH pulsatility that is responsible for male-specific liver activity and body growth.

**Mimicking GH Pulses Reverses the Feminized Phenotype of *Cry*<sup>-/-</sup> Males**—If our hypothesis is correct that altered GH profiles are responsible for the loss of sex dimorphism, it should be possible to reverse the hepatic feminization by reinstating GH pulses and interpulses in *Cry*<sup>-/-</sup> males. Accordingly, we administered bovine GH (50  $\mu$ g/mouse) at 12-h intervals during 1 week to *Cry*<sup>-/-</sup> males, a protocol previously shown to masculinize hepatic gene expression in wild type female mice (23). This treatment restores typical male *Mup1* mRNA levels and reverses the feminized pattern of hepatic *Cyp2b9*, *Cyp2d9*, *Cyp4a12*, *Cyp7b1*, and *Elovl3* in *Cry*<sup>-/-</sup> males to levels similar to those measured in untreated wild type males (Fig. 6). To exclude a spurious effect from high concentrations of exogenous GH, we forced the pituitary gland of *Cry*<sup>-/-</sup> males to secrete endogenous GH in a pulsatile manner but without generating excess GH exposure. Because patterned infusions of somatostatin can elicit rebound bursts of GH (36), we repeatedly injected octreotide (50 ng/mouse every 12 h), a long lasting somatostatin agonist, to *Cry*<sup>-/-</sup> males. This treatment also res-

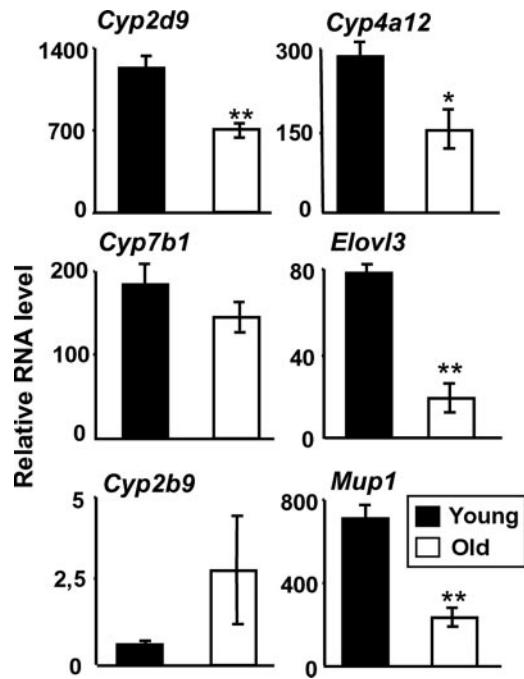


FIGURE 5. **Aged male mice have a feminized liver gene pattern.** Livers of 8-week-old (solid bars) and 2-year-old (open bars) C57BL/6 male mice were collected between ZT2 and ZT4. Sex-specific liver genes show a feminization of their expression level in aged males. Relative RNA levels are graphed as means  $\pm$  S.E.,  $n = 5$  animals in each condition. \*\*,  $p < 0.01$  and \*,  $p < 0.05$ , Mann-Whitney  $U$  test.

cues a male pattern of liver gene expression in the mutant mice (Fig. 6), and confirms that rhythmic activation of the GH axis, with regular and sustained low trough values of GH, is responsible for the reversal of the feminized phenotype of the  $Cry^{-/-}$  male liver.

## DISCUSSION

*Circadian Timekeeping as a Regulator of Ultradian GH Release*—Altogether, our data reveal *Cry1* and *Cry2* as genetic determinants of sexual dimorphism in liver metabolism. The presence of functional CRY products appears essential for tuning the ultradian GH pulsatility required for the male-specific liver activity. Similarly, previous studies reported that the circadian clock gene *Bmal1* is also necessary to maintain male-female differences in body growth (33) or that liver expression of *Elov13* follows a female-like profile in male homozygous *Clock/Clock* mutants (19). Moreover, male rats with lesioned SCN exhibit a feminized steroid metabolism (37), as we observed for  $Cry^{-/-}$  mice. Last, the alteration of circadian timekeeping during aging (34) is concomitant with dampening of the GH pulsatility (35) and leads to the feminization of liver gene expression in rats (27) and mice (this study). Thus, although we cannot exclude a non-circadian role for *Cry* genes, we conclude that the feminized phenotype of  $Cry^{-/-}$  males is at least partly due to the disruption of their circadian clock.

The SCN transiently synchronize the GH rhythm with the light-dark cycle, likely through somatostatinergic neurons (38, 39), and male rats with SCN lesions present alterations in the amplitude of ultradian GH pulses (39). Thus, the alteration of GH secretion in  $Cry^{-/-}$  may result from impaired circadian

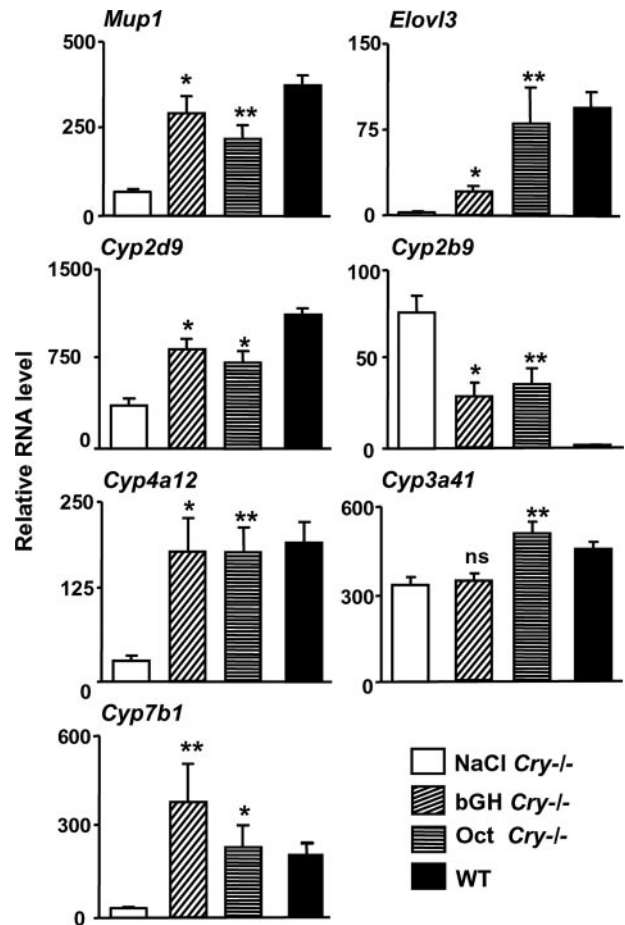


FIGURE 6. **Patterned GH pulses reverse the feminized phenotype of  $Cry^{-/-}$  males.** Liver gene expression was assessed at ZT4 in  $Cry^{-/-}$  males that received two subcutaneous injections a day, at ZT0 and ZT12 during 1 week, of saline ( $n = 4$ ), 50  $\mu$ g of highly purified pituitary bovine GH (bGH,  $n = 5$ ), or 50 ng of octreotide ( $n = 5$ ), and untreated control males ( $n = 5$ ). Both hormonal treatments restored a control-like expression pattern in the liver of  $Cry^{-/-}$  males. \*\*,  $p < 0.01$  and \*,  $p < 0.05$  as compared with NaCl, Mann-Whitney  $U$  test. WT, wild type.

outputs from their SCN (40). Indeed, the SCN, equipped with receptors for gonadal hormones (15, 16), may indirectly tune the GH secretion profiles responsible for dimorphic liver metabolism.

Another hypothesis, not exclusive from the former one, is that the circadian clockwork within the pituitary gland may synchronize unitary ultradian activities of GH-secreting cells (41, 42) through long distance homotypic cell networks (43, 44). Although such a possibility remains to be investigated, it is worth noting that circadian timekeeping is known to interfere with other rhythms outside the 24-h range, such as ultradian courtship song in flies (45) and infradian estrous cycles in mice (46).

The GH secretion profile in  $Cry^{-/-}$  males differs from that of control males, with fewer trough values. However, it remains distinct from the female GH pattern, which exhibits a lower mean level. Thus,  $Cry^{-/-}$  males have a unique GH profile that leads to a female-like metabolic pattern. However, this feminization is not complete and underscores the complex regulation of sexually dimorphic liver genes, which mainly involves transduction through the signal transducer and activator of tran-

## Sex Dimorphism in the Liver Depends on Circadian Timekeeping

scription 5b (STAT5b) and hepatic nuclear factor 4  $\alpha$  (HNF4 $\alpha$ ) pathways (47–49). For example, the expression level of the female-predominant gene *Cyp3a41* is not altered in *Cry*<sup>-/-</sup> mutants. Interestingly, several genes of the *Cyp3a* family, including *Cyp3a41*, were previously reported to be resilient to the inactivation of STAT5b (47) but not of HNF4 $\alpha$  (49). Our data may thus define a new paradigm to study the sexually dimorphic action of GH in the mammalian liver through both transduction cascades.

**Interrelationship between Circadian Timekeeping, Sex Dimorphism, and Liver Metabolism**—Finally, our data reveal that circadian timekeeping may control cell and body metabolism by pacing the secretion of pituitary GH, in addition to the well known direct regulation at the transcription level in liver cells (7, 8). Over the last decades, chronomedicine has been developed, assuming that a circadian control of sensitivity to drugs exists in humans as well as in rodents (7, 50, 51). Interestingly, a recent trial for time-modulated treatment of metastatic colorectal cancer revealed intriguing opposite outcomes for men and women patients (52). Further studies will be necessary to unveil whether our results could be extended to human beings and provide some mechanistic insights into how the circadian time, sex, and metabolism must interact and constitute an undividable troika to optimize personalized therapy.

**Acknowledgments**—We are indebted to A. F. Parlow (National Institutes of Health, NIDDK, National Hormone and Peptide Program, Torrance, CA) for providing us with bovine GH and reagents for radioimmunoassay. We thank C. Legerverend, F. Gachon, and A.-L. Mausset-Bonnefont for critical reading of the manuscript. D. Hadou, P. Makoundou, N. Oueslati, and S. Faibie are acknowledged for technical assistance.

### REFERENCES

- Rinn, J. L., Rozowsky, J. S., Laurenzi, I. J., Petersen, P. H., Zou, K., Zhong, W., Gerstein, M., and Snyder, M. (2004) *Dev. Cell* **6**, 791–800
- Yang, X., Schadt, E. E., Wang, S., Wang, H., Arnold, A. P., Ingram-Drake, L., Drake, T. A., and Lusis, A. J. (2006) *Genome Res.* **16**, 995–1004
- Ahluwalia, A., Clodfelter, K. H., and Waxman, D. J. (2004) *Mol. Endocrinol.* **18**, 747–760
- Rinn, J. L., and Snyder, M. (2005) *Trends Genet.* **21**, 298–305
- Isensee, J., and Ruiz Noppinger, P. (2007) *Gend. Med.* **4**, Suppl. B, S75–S95
- Schwartz, J. B. (2003) *Clin. Pharmacokinet.* **42**, 107–121
- Gachon, F., Olela, F. F., Schaad, O., Descombes, P., and Schibler, U. (2006) *Cell Metab.* **4**, 25–36
- Panda, S., Antoch, M. P., Miller, B. H., Su, A. I., Schook, A. B., Straume, M., Schultz, P. G., Kay, S. A., Takahashi, J. S., and Hogenesch, J. B. (2002) *Cell* **109**, 307–320
- Zhang, Y. K., Yeager, R. L., and Klaassen, C. D. (2008) *Drug Metab. Dispos.* **37**, 106–115
- Kornmann, B., Schaad, O., Bujard, H., Takahashi, J. S., and Schibler, U. (2007) *PLoS Biol.* **5**, e34
- Lamia, K. A., Storch, K. F., and Weitz, C. J. (2008) *Proc. Natl. Acad. Sci. U. S. A.* **105**, 15172–15177
- Daan, S., Damassa, D., Pittendrigh, C. S., and Smith, E. R. (1975) *Proc. Natl. Acad. Sci. U. S. A.* **72**, 3744–3747
- Davis, F. C., Darrow, J. M., and Menaker, M. (1983) *Am. J. Physiol.* **244**, R93–R105
- Roenneberg, T., Kuehne, T., Pramstaller, P. P., Ricken, J., Havel, M., Guth, A., and Merrow, M. (2004) *Curr. Biol.* **14**, R1038–1039
- Karatsoreos, I. N., Wang, A., Sasanian, J., and Silver, R. (2007) *Endocrinology* **148**, 5487–5495
- Vida, B., Hrabovszky, E., Kalamatianos, T., Coen, C. W., Liposits, Z., and Kallo, I. (2008) *J. Neuroendocrinol.* **20**, 1270–1277
- van der Horst, G. T., Muijtjens, M., Kobayashi, K., Takano, R., Kanno, S., Takao, M., de Wit, J., Verkerk, A., Eker, A. P., van Leenen, D., Buijs, R., Bootsma, D., Hoeijmakers, J. H., and Yasui, A. (1999) *Nature* **398**, 627–630
- Reinerink, E. J., Doorn, L., Jansen, E. H., and Van Iersel, A. A. (1991) *J. Chromatogr.* **553**, 233–241
- Anzulovich, A., Mir, A., Brewer, M., Ferreyra, G., Vinson, C., and Baler, R. (2006) *J. Lipid Res.* **47**, 2690–2700
- Brolinson, A., Fourcade, S., Jakobsson, A., Pujol, A., and Jacobsson, A. (2008) *Endocrinology* **149**, 3158–3166
- Alvarez, J. D., Hansen, A., Ord, T., Bebas, P., Chappell, P. E., Giebultowicz, J. M., Williams, C., Moss, S., and Sehgal, A. (2008) *J. Biol. Rhythms* **23**, 26–36
- Legerverend, C., Mode, A., Wells, T., Robinson, I., and Gustafsson, J. A. (1992) *FASEB J.* **6**, 711–718
- Jarukamjorn, K., Sakuma, T., Jaruchotikamol, A., Ishino, Y., Oguro, M., and Nemoto, N. (2006) *Toxicology* **219**, 97–105
- Waxman, D. J., and O'Connor, C. (2006) *Mol. Endocrinol.* **20**, 2613–2629
- MacLeod, J. N., Pampori, N. A., and Shapiro, B. H. (1991) *J. Endocrinol.* **131**, 395–399
- Tannenbaum, G. S., and Martin, J. B. (1976) *Endocrinology* **98**, 562–570
- Dhir, R. N., and Shapiro, B. H. (2003) *Proc. Natl. Acad. Sci. U. S. A.* **100**, 15224–15228
- Clark, R. G., Jansson, J. O., Isaksson, O., and Robinson, I. C. (1985) *J. Endocrinol.* **104**, 53–61
- Low, M. J., Otero-Corchon, V., Parlow, A. F., Ramirez, J. L., Kumar, U., Patel, Y. C., and Rubinstein, M. (2001) *J. Clin. Investig.* **107**, 1571–1580
- Norstedt, G., and Palmiter, R. (1984) *Cell* **36**, 805–812
- Kondratov, R. V., Kondratova, A. A., Gorbacheva, V. Y., Vykhovanets, O. V., and Antoch, M. P. (2006) *Genes Dev.* **20**, 1868–1873
- Masaki, S., Todo, T., Nakano, Y., Okamura, H., and Nose, H. (2005) *J. Physiol. (Lond.)* **566**, 213–224
- Sun, Y., Yang, Z., Niu, Z., Wang, W., Peng, J., Li, Q., Ma, M. Y., and Zhao, Y. (2006) *J. Biomed. Sci.* **13**, 845–851
- Hastings, M. H., Reddy, A. B., and Maywood, E. S. (2003) *Nat. Rev. Neurosci.* **4**, 649–661
- Kuwahara, S., Sari, D. K., Tsukamoto, Y., Tanaka, S., and Sasaki, F. (2004) *Brain Res.* **998**, 164–173
- Clark, R. G., and Robinson, I. C. (1988) *Endocrinology* **122**, 2675–2682
- Gustafsson, J. A., Eneroth, P., Hokfelt, T., and Skett, P. (1978) *Endocrinology* **103**, 141–151
- Davies, J. S., Carter, D. A., and Wells, T. (2004) *Endocrinology* **145**, 2950–2958
- Willoughby, J. O., and Martin, J. B. (1978) *Brain Res.* **151**, 413–417
- Albus, H., Bonnefont, X., Chaves, I., Yasui, A., Doczy, J., van der Horst, G. T., and Meijer, J. H. (2002) *Curr. Biol.* **12**, 1130–1133
- Bonnefont, X., Fiekers, J., Creff, A., and Mollard, P. (2000) *Endocrinology* **141**, 868–875
- Bonnefont, X., and Mollard, P. (2003) *FEBS Lett.* **548**, 49–52
- Bonnefont, X., Lacampagne, A., Sanchez-Hormigo, A., Fino, E., Creff, A., Mathieu, M. N., Smallwood, S., Carmignac, D., Fontanaud, P., Travo, P., Alonso, G., Courtois-Coutry, N., Pincus, S. M., Robinson, I. C., and Mollard, P. (2005) *Proc. Natl. Acad. Sci. U. S. A.* **102**, 16880–16885
- Fauquier, T., Guérineau, N. C., McKinney, R. A., Bauer, K., and Mollard, P. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98**, 8891–8896
- Kyriacou, C. P., and Hall, J. C. (1980) *Proc. Natl. Acad. Sci. U. S. A.* **77**, 6729–6733
- Miller, B. H., Olson, S. L., Turek, F. W., Levine, J. E., Horton, T. H., and Takahashi, J. S. (2004) *Curr. Biol.* **14**, 1367–1373
- Clodfelter, K. H., Holloway, M. G., Hodor, P., Park, S. H., Ray, W. J., and Waxman, D. J. (2006) *Mol. Endocrinol.* **20**, 1333–1351
- Holloway, M. G., Laz, E. V., and Waxman, D. J. (2006) *Mol. Endocrinol.* **20**,

## Sex Dimorphism in the Liver Depends on Circadian Timekeeping

- 647–660
49. Wiwi, C. A., Gupte, M., and Waxman, D. J. (2004) *Mol. Endocrinol.* **18**, 1975–1987
50. Gorbacheva, V. Y., Kondratov, R. V., Zhang, R., Cherukuri, S., Gudkov, A. V., Takahashi, J. S., and Antoch, M. P. (2005) *Proc. Natl. Acad. Sci. U. S. A.* **102**, 3407–3412
51. Levi, F., and Schibler, U. (2007) *Annu. Rev. Pharmacol. Toxicol.* **47**, 593–628
52. Giacchetti, S., Bjarnason, G., Garufi, C., Genet, D., Iacobelli, S., Tampellini, M., Smaaland, R., Focan, C., Coudert, B., Humblet, Y., Canon, J. L., Adenis, A., Lo Re, G., Carvalho, C., Schueller, J., Anciaux, N., Lentz, M. A., Baron, B., Gorlia, T., and Levi, F. (2006) *J. Clin. Oncol.* **24**, 3562–3569