Studies on the effects of calorie restriction on the gene expression in *Brachionus plicatilis*

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Dedication

To all my family: my father, my mother, my elder brother, my two younger brothers, and my youngest sister — for their enormous support and sacrifices enabled me to devote myself to my career.

Statement of Originality

I do hereby declare that this submission is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person nor material which to a substantial extent has been accepted for the award of any other degree or diploma of the university or other institute of higher learning, expect where due acknowledgment has been made.

Aung Kyaw Swar Oo

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List of Publications

Parts of works described in this dissertation have been published in the following journals:

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Contents

Abbreviations

14-3-3 : Multifunctional 14-3-3 family chaperone

16S rRNA : Mitochondrial 16S rRNA

αAmy : Alpha amylase

Amy2 : Amylase 2

AMPK : AMP-activated protein kinase

BAS : Brujewicz artificial seawater

BCCIPβ : BRCA2 and CDKN1A-interacting protein, isoform BCCIP beta

BrdU : 5-bromo-2′-deoxyuridine

CaM : Calmodulin

CaM64B : Calmodulin (synthetic construct)

cDNA : Complementary DNA

CDT1 : CDT1 protein

CHP : Conserved hypothetical protein

COX II : Cytochrome *c* oxidase subunit II

CR : Calorie restriction or calorie-restricted

DAPI : 4', 6-diamidino-2-phenylindole

Dnahc3 : Dynein heavy chain domain 3

EBF : EBF protein

EF-1α : Elongation factor-1α

Ef-tu : Elongation factor, tubulin

FOXO : Forkhead box O, the forkhead family of transcription factor

Gale : Galactose-4-epimerase, UDP

Glb : β-galactosidase

Glase : Glycogen phosphorylase

IPS2 : 2-isopropylmalate synthase

IRE : Iron-responsive element

Irp : Iron regulatory protein

Lis1 : Lissencephaly-1

Mn-SOD : Manganese-superoxide dismutase

Msh6p : Mismatch repair protein in mitosis and meiosis

mtPP : Mitochondrial processing peptidase beta subunit

Notch : Neurogenic gene encodes a large transmembrane protein

NSUN2 : NOL1/NOP2/Sun domain family 2 protein

PBS : Phosphate-buffered saline

PFA : Paraformaldehyde

Phm : Peptidylglycine-hydroxylating monooxygenase

PK-TRP : Serine/threonine protein kinase with TRP repeats

Pole : DNA polymerase epsilon

Pols : DNA polymerase sigma

PS/PGp/Cl : Phosphatidylserine/phosphatidylglycophosphate/cardiolipin synthase

qRT-PCR : Quantitative real-time RT-PCR

ROS : Reactive oxygen species

rpt : Proteasome regulatory particle

RT-PCR : Semi-quantitative reverse transcription-PCR

SDHD : Succinate dehydrogenase complex subunit D

Ser : Serine protease

SOD : Supreoxide dismutase

Spnb1 : Spectrin beta chain

SSH : Suppression subtractive hybridization

stom : Stom protein

STPP : Serine/threonine phosphatase

Stt3 : Oligosaccharyl transferase STT3 subunit homolog

TFPI : Tissue factor pathway inhibitor

TOR :Target of rapamycin

Tsase : Transposase

tub : Beta 2 tubulin

UTR : Untranslated region

WF : Well feeding or well-fed

Wnt : Wingless protein, a secreted morphogen

Znf : Zinc finger protein

List of Tables

List of Figures

General introduction

Calorie restriction (CR, a dietary intervention without malnutrition) is the only intervention known to date that consistently decreases the biological rate of aging and increases both mean and maximum life spans. Since the initial report by McCay *et al*., (1935) in which dietary restriction increases the maximum life span of laboratory rats, many studies have been focused on CR and its benefits to a wide range of organisms. Subsequent studies have confirmed this result that CR typically increases 20–30% in both mean and maximum life span (Weindruch and Walford, 1988; Sprott, 1997). Life span extension through CR is not confined to rodents, and it has been a widespread phenomenon that CR increases maximum life span of other species including nematodes (Van Voorhies and Ward, 1999), insects (Carey, 2003; Mair *et al.*, 2003), water flea, spider, and fish (Weindruch and Walford, 1988). Overall, CR extends life span across a diverse range of vertebrates and invertebrates (Klass, 1977; Chapman and Partridge, 1996; Carey *et al.*, 1999; Lane *et al.*, 2000; Lin *et al.*, 2000), suggesting that the mechanism of CR-induced life span extension is evolutionarily conserved. Studies on nonhuman primates have begun and, although it will take several more years to obtain evidence of life span alterations, some changes in physiological profiles similar to those observed in rodents have been reported (Roth *et al*., 1995; Lane *et al*., 1996). Ongoing studies suggest that the CR-related reduction in the aging rate reported in rodents and other short-lived species could also take place in primates (Roth *et al*., 2004) and thus possibly in humans. However, while findings on other species can suggest possible mechanisms that are relevant to humans, the only way to assert whether CR works in human is to conduct studies on human itself. Although full

longevity data for human are not yet available, studies have reported that CR reduces the risk of developing diseases, such as diabetes and atherosclerosis (Fontana *et al*., 2004; Larson-Meyer *et al*., 2006), and induces a number of the same adaptive responses that occur in CR laboratory animals.

Restricted feeding regimens which successfully extend survival of the CR animals are needed to be designed to avoid malnutrition. The method of imposing CR on laboratory animals has varied between studies. Some have restricted food to a fixed proportion of the *ad libitum* levels; others have established a level of food intake that maintains a fixed, but reduced, body weight (Merry, 1995). The essential requirement is that total energy intake is decreased to a particular level that can operate an error-free metabolic network in CR animals. It has been observed that varying the proportions of specific dietary components, such as protein and carbohydrate, has little effect on CR animals (Kristal and Yu, 1994), indicating the diet does not lack any essential nutrient. Restriction of fat, protein or carbohydrate alone, without an overall reduction in total energy intake, does not work for extending survival in rodent species (Dalderup and Visser, 1969; Birt *et al*., 1982; Feldman *et al*., 1982). Although various CR regimens are used in different organisms, the effect on life span by CR is remarkably similar in each case (reviewed in Kennedy *et al.*, 2007). Typically, animals that have a 40% reduction in overall calorie intake, or that are maintained at 50% of the body weight of animals fed *ad libitum*, show life span extension by 20–40% (Weindruch and Walford, 1988; Merry, 1995).

The effects of CR might be specific to gender of organisms and time at which CR is imposed. It has been reported that life span extension by CR is more effective in females than males of fruitfly *Drosophila melanogaster* (Magwere *et al.*, 2004),

Mediterranean fruit fly *Ceratitis capitata* (Davies *et al.*, 2005), medflies (Carey *et al.*, 1999), and silkworm *Bombyx mori* (Li *et al.*, 2009). To be effective in extending survival, CR has to be imposed during the postweaning life span. Restricted feeding before weaning conveys no additional advantage and even is detrimental to subsequent survival. CR in juveniles has a detrimental effect on growth and development (Merry, 1995). Tu and Tatar (2003) reported that diet restriction on the larvae of *D. melanogaster* can affect the subsequent body size and fecundity of adults, but not the life span. The longer the period of CR in adults, the greater the effect on longevity (Kristal and Yu, 1994). However, there are some controversies over the beneficial effects of CR in regard to animal age at which CR is imposed. There is some evidence to suggest that a period of restriction early in life can have lasting effects on aging and life span (Ozanne *et al*., 2004, 2005). Yu *et al*. (1985) reported that even a brief (4.5 months) CR started at 6 weeks of age in rats produced a 15% increase in life span. Although it has been reported that CR could be ineffective or even detrimental if started late in life (Forster *et al.*, 2003), various studies show that CR could exert its positive effects even in middle age or later (Lee *et al.*, 2002; reviewed in Takahashi and Goto, 2002). It has been reported that both short- and long-term CR in older rhesus monkeys may have beneficial effects on certain risk factors associated with diabetes or cardiovascular disease or both (Bodkin *et al.*, 1995; Lane *et al.*, 2000).

The effects of CR seem to be reversible, in that cessation of CR results in a rapid return to the normal state (reviewed in Spindler, 2005), with a concomitant reduction in life span relative to animals maintained on lifelong CR (Merry *et al*., 2008). Refeeding previously calorie-restricted animals is detrimental to subsequent survival, suggesting that the mechanism through which diet acts to retard aging is both dynamic

and reversible (Merry, 1987). However, the age at which the switch from CR back to normal feeding occurs may be important. Yu and colleagues demonstrated that 4.5 months of CR, from 1.5 to 6 months of age, in male F344 rats was sufficient to significantly extend both median and maximum life span in conjunction with a delayed onset of age-related disease (Maeda *et al*., 1985; Yu *et al.*, 1985), and similar results were observed in other rat strains (Nolen, 1972; Ross, 1972). These studies suggest that CR-induced life span extension may depend on both the timing and duration of the period of dietary intervention.

As CR is the most effective intervention known to extend life span in a variety of species including mammals (Weindruch and Walford, 1988; Roth *et al.*, 2001) and the mechanism of aging upon which CR is acting is evolutionary conserved (Partridge and Gems, 2002), understanding the mechanisms that mediate the beneficial effects of CR on longevity and age-dependent traits will help harness the benefits of CR. At present, there are still many debates over the mechanisms that would be responsible for how CR works. Over the past 70 years at least 10 different hypotheses have been put forth to explain how CR works, but almost all of them have been found to be contradictory and the remainder fails to explain numerous observations about CR and life span expansion (Sinclair, 2005). Some early hypotheses are about developmental delays and reduced metabolic rates. Current hypotheses concern insulin/IGF-1 signaling pathway, target of rapamycin (TOR) pathway, reduced reactive oxygen species, cell survival hypothesis, protein turnover, glucocorticoid cascade, and the hormesis hypothesis (Sinclair, 2005). Although these hypotheses have their own stand on approaches to mechanisms of how CR works, they are in someway overlapping each other at the molecular level. Wnt and Notch signaling pathway has been implicated in

developmental processes of organisms, and CR modulates the expression of a number of genes that participate in the animal development. Generally, genes related to Wnt signaling pathway are down-regulated by CR (Wu *et al.*, 2009). AMP-activated protein kinase (AMPK) is well known to regulate lipid metabolism and protein synthesis in response to low energy to restore energy levels (reviewed in Kahn *et al.*, 2005). In nematode *Caenorhabditis elegans*, AMPK is necessary for life span extension in response to a developed dietary restriction regimen (Greer *et al.*, 2007). An energy-sensing AMPK-FOXO pathway mediates life span extension induced by a novel method of dietary restriction in *C. elegans* (Greer *et al.*, 2007, 2009). However, several studies using other dietary restriction regimens showed that longevity in worms was not dependent on a FoxO/*daf*-16 pathway (Lakowski and Hekimi, 1998; Houthoofd *et al.*, 2003; Panowski *et al*., 2007). Particularly, the modification of insulin/IGF-I signaling pathway in CR animals has been considered of special interest, as this highly conserved system has been proposed to regulate longevity in many animals from nematodes to mammals (reviewed in Gems and Partridge, 2001). Functional mutation of single genes in that pathway extends the maximum life span in mice (reviewed in Bartke and Brwon-Borg, 2004), and these mutant mice showed some physiological characteristics similar to those of CR rodents. However, it has been reported that CR extends the maximum life span of one of those mutant mice, Ames dwarfs (Bartke *et al*., 2001), suggesting that the pathways responsible for increasing longevity can be different in the mutants and restricted animals. In flies, CR did not further extend the life span of long-lived *chico* (encodes an insulin receptor substrate) mutants (reviewed in Partridge *et al.*, 2005). In contrast to these results, CR did not extend the life span of worms through insulin/IGF-I signaling pathway (Houthoofd *et al.*, 2003). Another nutrient

responsive pathway that is conserved in higher eukaryotes and has interconnections with insulin/PI3K pathway is TOR pathway. TOR controls cell growth and proliferation through impinging on a diverse array of cellular processes at the level of transcription, translation and intracellular trafficking (reviewed in Schmelzle and Hall, 2000; reviewed in Jacinto and Hall, 2003). Prolonged treatment with rapamycin or TOR deletion in yeast caused phenotypic changed characteristic of starved (G_0) cells: altered transcription pattern, down-regulation of protein synthesis, accumulation of storage carbohydrates and acquisition of thermo-tolerance (Barbet *et al*., 1996). It has been reported that life span in organisms is extended by reducing the TOR signaling activity (Kapahi *et al*., 2004; Kaeberlein *et al*., 2005; Powers *et al*., 2006). Inhibiting TOR by rapamycin treatment up-regulated mitochondrial respiration by inducing the expression of genes encoding TCA cycle enzymes and proteins involved in oxidative phosphorylation (Hardwick *et al*., 1999; Shamji *et al*., 2000). Similar expression pattern might occur under CR because it has been reported that CR promoted mitochondrial biogenesis and respiration in mice (Nisoli *et al*., 2005). These findings also suggest that TOR signaling pathway under CR is likely to participate in metabolic processes and cell survival through the regulation of reactive oxygen species (ROS) production by mitochondrial biogenesis. Physiological changes in CR animals have been reported, including alterations in hormonal pathways. Glucocorticoid levels were enhanced in CR animals and its increment during CR has been proposed to have an important role in the beneficial effects of CR (Sabatino *et al*., 1991). Hormesis hypothesis of CR proposed that CR represents a low-intensity biological stress to the organism which elicits a defense response that helps protect it against the causes of aging (reviewed in Masoro, 2007). It seems that the effect of CR is too complex to be described by a single

hypothesis.

CR induces diverse metabolic changes in organisms, and it is currently unclear whether and how these metabolic changes affect life span extension. One hypothesis for the mechanism of life span extension in response to CR is that organisms have evolved a dynamic resource-allocation system in which energy is transferred from reproduction to somatic maintenance (Kirkwood, 2005). Resources allocated to reproduction are used to produce progeny, whereas resources allocated to somatic maintenance are used to conserve state. Energy allocated to somatic maintenance is used for anti-aging cellular functions such as DNA repair and free radical scavenging (Blaxter, 1989). The potential benefit is that animal gains an increased chance of survival with a reduced intrinsic rate of senescence, thereby permitting reproductive value to be preserved until the famine is over. An increase in life span at the expense of reproduction is seen as a strategy to tolerate periods of food deprivation so that animals can start reproduction again when food is in a higher supply (Weithoff, 2007). Extension of life span during CR is often associated with the suppression of reproduction, although there is some variation depending on species, sex, the degree of restriction, and the age at which restriction is first applied (Weindruch and Walford, 1988). A comprehensive comparative study using nine rotifer species well demonstrated the discrepancies in life history responses to periods of food shortage; some species continued reproduction in periods of food shortage with a decrease in their life spans, whereas other species increased their life spans with a decrease in their reproduction (Kirk, 2001). Weithoff (2007) studied the effect of dietary restriction on life span as well as reproduction in two rotifer species, *Cephalodella* sp. and *Elosa worallii,* and reported that only *E. worallii* responded to dietary restriction with an increase in life span at the expense of reproduction.

Reproductive rate and status are another important factors that have been shown to alter longevity in invertebrates and mammals (Bell *et al.*, 1986; Holliday *et al*., 1989; Masoro *et al.*, 1996). For example, female *D. melanogaster* with environmentally (Partridge *et al*., 1987) or genetically (Sgrò and Partridge, 1999) elevated rates of egg production have reduced life span as compared to controls. Thus, the mechanisms involved in life span extension of CR organisms still remain unclear and need clarification.

Background

The rotifer *Brachionus plicatilis* has been a well-known model organism and used in CR studies. *B. plicatilis* is a small zooplankton approximately 0.3 mm in length that inhabits brackish waters. It is an economically important species as a live food organism in aquaculture (Hagiwara *et al*., 2001), because it has a rich nutritional profile and a suitable size for larval fish and shrimp. It reproduces either sexually or asexually with one of the highest population growth rate among metazoans (Pourriot and Snell, 1983; Nogrady *et al*., 1993). There are several reports on obligate parthenogenetic strains (reproduce only asexually) in *Brachionus* sp. (Bennett and Boraas, 1989; Fussmann *et al*., 2003) and Ishikawa strain of *B. plicatilis* has been known to reproduce asexually: mictic females or males are not observed (Yoshinaga *et al*., 2000). Thus, it is a useful model for studies on genetic mechanisms related to life history alterations, because obligatively asexual population typically consists of nearly identical genotypes and shows excess or fixed heterozygosity at several loci (Innes *et al*., 1986; Ward *et al*., 1994), possibly lacking the genetic variation.

There are several reports on CR-induced changes in life history parameters of *B. plicatilis*. The rotifer subjected to CR in a feeding schedule of 3 h/day showed its life span two times longer than that well fed, and offspring production concomitantly decreased about ten times under CR (Yoshinaga *et al*., 2003). Thus, CR is one of the biological factors that shift a mode of reproduction and concomitantly a mode of life span in the rotifer. Such trade-off between lifetime fecundity and life span is proposed as an alternative life history strategy of rotifers under starved conditions (Snell and King, 1977; Kirk, 1997; Yoshinaga *et al*., 2000; Weithoff, 2007). It is also proposed that the

effects of CR on the starvation tolerance are transmitted from parents to their offspring in the rotifer *B. plicatilis* (Yoshinaga *et al*., 2001). These life history alterations in response to CR are likely to occur during population growth, possibly affecting the population dynamics of the rotifer.

Molecular approaches to the effects of CR on life span have been conducted using rotifer as a model organism. It is widely accepted that life span is regulated by the interaction between oxidative stress and an enzymatic antioxidation (Orr and Sohal, 1994; Parkes *et al*., 1998; reviewed in Finkel and Holbrook, 2000). The roles of antioxidants in life span extension have been reported in the rotifer *Asplanchna brightwelli* (Sawada and Enesco, 1984; Bozovic and Enesco, 1986). The major antioxidant enzyme, superoxide dismutase (SOD), catalyzes decomposition of ROS, which provokes massive damages to DNA, proteins, and lipids. The accumulated mRNA levels of manganese-SOD (Mn-SOD), which functions in mitochondria, were found to increase in calorie-restricted, long-lived rotifer *B. plicatilis* (Kaneko *et al*., 2005). On the other hand, it has been claimed that the dietary restriction in rotifer *A. brightwelli* retarded the rate of nuclear division in the gastric glands and vitellarium (yolk-secreting gland) (Verdone-Smith and Enesco, 1982) and that CR decreased cell proliferation in rat (Lok *et al*., 1990; Lu *et al*., 1993, 2002). Although it is generally accepted that an energy saving through the suppression of reproduction during the period of food shortage is a prerequisite to a longer life span, little is known at present about the genes expressed under CR.

Rationale and objectives

Organisms living in various environments, where both biotic and abiotic factors vary over time, need abilities to overcome the daily challenges. In order to accomplish this requirement, the organisms must have well-developed biological systems that can sense changes in the environment and that can adjust the body metabolism to the changes. In fact, organismal metabolic processes are tightly and specifically controlled at the molecular level by proper gene expression. Understanding the gene expression of organisms in response to changes in their environments will give people insights into the populations in the real world, and this understanding is a prerequisite for managing the populations of ecologically and/or commercially important species in both laboratory and nature. In the wild, studies on a specific gene expression in response to a particular factor seem difficult and hard to draw a reliable conclusion on the functional importance of the expressed genes in regard to the factor, because any other factors might also exert their effects during the investigation. Such kinds of impediment can be overcome under laboratory conditions in which by using a suitable model animal a specific gene expression in response to a particular factor of interest can be detected.

Some animals live in environments in which the availability of food resources is unstable. When food is scarce the animals suppress reproduction, but invest resources into survival until food is again available, and they can reproduce. Under these circumstances their life span can be increased. This has been universally accepted CR-induced beneficial effects in most, but not all, organisms. A laboratory method of CR, in some way analogous to a scarcity of food in the wild, has been known to extend

life span in a variety of species including mammals (Weindruch and Walford, 1988; and Roth *et al.*, 2001), and CR-induced mechanisms are also known to be conserved in a wide range of species (Partridge and Gems, 2002). Thus, the beneficial effects of CR observed in a simple organism under laboratory conditions could also be exploited for other higher organisms including human.

The rotifer *B. plicatilis*, a simple eukaryotic zooplankton, is an ecologically and commercially important species, and has been used as a model organism in various fields such as population dynamics, ecotoxicology and aging. Many environmental factors fluctuate the rotifer populations in nature through changes in the life history parameters of individual rotifers within populations. *B. plicatilis* shows similar life history alterations that are also observed in other higher organisms in response to CR. Although many studies have been conducted to identify environmental factors that influence rotifer populations, the molecular mechanisms involved still remain to be elucidated. As a first attempt to study molecular mechanisms, gene(s) differentially expressed by CR in the rotifer was analyzed under different experimental conditions in this study.

Synopsis of chapters

In Chapter 1, calorie restriction (CR)-induced differentially expressed genes were investigated in the parthenogenetic rotifer *Brachionus plicatilis* (Ishikawa strain). Eggs were collected from the stock cultures and hatched out. Neonates were divided into two groups: one for well-feeding (WF, fed *ad libitum*) as the control and the other for calorie restriction (CR, fed 3/h day). cDNA subtraction was carried out by suppression subtractive hybridization (SSH) technique. cDNAs synthesized from the CR sample were used as a tester, whereas cDNAs constructed from the WF sample were used as a driver. The tester cDNAs were subtracted from the driver cDNAs. The subtracted PCR products were ligated into the pGEM-T plasmid vectors and the plasmids were transformed into *Escherichia coli*. Afterward, the purified, inserted plasmid DNAs were sequenced. Briefly, revised expressed sequence tags (ESTs) were submitted to the NCBI database for searching any known gene counterparts with homologous sequences in the database. Sequence annotation by Gene ontology revealed 6 functional groups and one unclassified group among 38 differentially expressed genes.

SSH itself is a powerful technique to isolate the differentially expressed genes, but sometime it may yield false results and thus some additional methods are still necessary to verify the efficiency of SSH. Therefore, 38 differentially expressed genes were further validated two times by semi-quantitative reverse transcription (RT)-PCR (RT-PCR). Among 38 differentially expressed genes obtained by SSH, RT-PCR in two experiments confirmed that while 29 genes were consistently up-regulated in the CR groups, 8 genes were consistently down-regulated in the same groups. Other one gene showed its unchangeable mRNA levels in the CR and WF groups. Rotifer elongation

factor-1α gene (*EF-1α*) was used as an internal standard in comparing the relative mRNA levels of the target genes.

In Chapter 2, 17 genes were selected from 38 differentially expressed genes and their expressions were further studies by more accurate quantitative real-time reverse transcription-PCR (qRT-PCR). Gene were selected based first on their putative functions and second on their sequence lengths desirable for designing qRT-PCR primers. The selected genes generally encompassed 4 groups according to Gene ontology: 1) genes related to DNA synthesis, 2) genes related to cellular structure, transport and division, 3) genes related to metabolism, and 4) genes related to other functions. Group 1 comprised 4 genes encoding *Msh6p*, *Pole*, *CDT1*, and *Pols*. Group 2 consisted of 5 genes encoding *BCCIPβ*, *Lis1*, *Dnahc3*, *Spnb1*, and *CaM64B*. Group 3 included 4 genes encoding *stom*, *SDHD*, *Irp*, and *Amy2*. Group 4 had 4 genes encoding *TFPI*, *Tsase*, *16S rRNA*, and *14-3-3*.

First, expression levels were studied in the CR and WF groups containing individuals of various ages. Second, the expression levels were studied in the stationary and exponential phases of population growth. The first experiment aimed to detect the expression levels of the selected genes at several sampling points, and to understand their expression patterns in the CR and WF groups of various ages. The same feeding regimen used in the cDNA subtraction was used; the CR group was fed 3 h/day, whereas the WF group was fed *ad libitum*. Sampling was carried out for 8 consecutive days. Relative mRNA levels of the target genes were determined by a comparative Ct method with reference to that of *EF-1α* gene as the internal control and the normalized Ct values were compared between the CR and WF groups.

Expression patterns in the CR and WF conditions were considered up- or down-regulated if at least $p<0.05$ significance level was observed on two consecutive culture days. By this assessment, while 16 selected genes could be verified their up-regulation, one gene, *Spnb1*, showed a propensity for both up- and down-regulation in the CR rotifer population of various age compositions.

The second experiment was intended to detect the mRNA expression levels of the selected genes in rotifers at exponential and stationary phases mimicking WF and CR, respectively. Sampling was conducted when the population had entered the exponential and stationary phases during population growth. The mRNA levels were detected by qRT-PCR and the levels were compared between the two phases according to the same procedures as described above.

Expression patterns in rotifers at the exponential and stationary phases revealed that while 14 selected genes were significantly up-regulated in the stationary phase, other 3 genes, *CDT1*, *Pols* and *Spnb1*, showed their down-regulation in the same phase, but significance level was observed only for *CDT1*. After assembling the expression patterns, 14 out of 17 selected genes showed a common up-regulated pattern during both CR and CR-like stationary phase. Of other 3 genes, *CDT1* and *Pols* contradicted their expressions between CR and CR-like stationary phase. *Spnb1* expression pattern remained ambiguous.

Finally, the expression patterns during CR were examined in comparison with those during the stationary phase. Because CR-like phenomena might occur in the stationary phase, genes with their consistent up and down expression patterns during CR and CR-like stationary phase would give information about whether their expressions are directly regulated by a limited food condition.

In Chapter 3, DNA synthesis and more detail expression patterns of the selected genes were studied in the CR and WF groups of the same ages. Because rotifers are eutelic (animals with about 1,000 total cells in the adult form) and organ-specific nuclear division rate in rotifers was retarded by dietary restriction, DNA synthesis- and cell cycle-related genes were give priority in this Chapter. The same 3 h/day feeding regimen was used for the CR group, whereas the WF group was fed *ad libitum*. DNA synthesis in the rotifers was detected by BrdU labeling. The expression levels of selected genes were also analyzed in rotifers from 0 (egg) to 33 hph stages by qRT-PCR.

BrdU labeling showed that the BrdU labels together with the vitellarium volumes were gradually increased in the WF rotifers, indicating that DNA was increasingly synthesized in the vitellarium of the WF rotifers. In contrast to the WF counterparts, the BrdU labels as well as the vitelalrium volumes were not conspicuous in the CR rotifers, indicating that CR suppresses vitellarium DNA synthesis in the CR rotifers.

Regarding to the gene expression, 13 genes showed significant up-regulation in the CR rotifers. Three genes, *Pols*, *Lis1* and *14-3-3*, showed their up-regulation in the CR rotifers during the period of 9 to 21 hph stages, afterward their levels were up-regulated in the WF rotifers. *CDT1* was significantly up-regulated in the WF rotifers, especially during the age of reproduction, and its up-regulation was time-dependent. Based on their putative functions and their expression patterns in the present study, *CDT1*, *BCCIPβ* and *Lis1* are assumed to be most responsible for the reproductive performance of the rotifer *B. plicatilis* in response to the variability of food resources.

Finally, Chapter 4 is culminated with the general discussion based on the

present study to highlight the genes which are most responsible for the reproductive performance of rotifers under CR. Although discussions largely deal with the expression patterns of DNA synthesis- and cell cycle-related genes in comparison with the results of BrdU labeling, the importance of some other genes are also included.

CHAPTER 1

Identification of genes differentially expressed by calorie restriction in the rotifer *Brachionus plicatilis*

The rotifer *B. plicatilis* Ishikawa strain was used in this study. Because it has been known to reproduce asexually (mictic females or males are not observed; Yoshinaga *et al.*, 2000), using this species might eliminate any sex-related differences in gene expression. It is also a useful model for studies on genetic mechanisms related to life history alterations, as obligatively asexual populations typically consist of nearly identical genotypes and shows excess of fixed heterozygosity at several loci (Innes *et al.*, 1986; Ward et al., 1994), possibly lacking the genetic variation. Thus, it is most likely that using this species would provide a stable gene expression under CR and other conditions as well.

Section 1: cDNA subtraction

Suppression subtractive hybridization (SSH) is a powerful and reliable technique to identify differentially expressed genes that are involved in physiological processes of both aquatic invertebrates (Brown *et al.*, 2006; Soetaert *et al.*, 2006) and vertebrates (Reynders *et al.*, 2006; Wang and Wu, 2007) responding to various environmental conditions. In this section, SSH was used to identify differentially expressed genes in calorie-restricted rotifer *B. plicatilis*.

Materials and methods

Animal culture and sample preparation

The parthenogenetic rotifer *B. plicatilis* Ishikawa strain, which is phylogenetically closer to *B. manjavacas* than to *B. plicatilis* sensu stricto (Yoshinaga *et al*., 2004), was used in this study. Rotifer cultures were performed using Brujewicz artificial seawater (BAS) (Subow, 1931; salinity 33 ppt, sterilized by 0.45-μm filter) consisting of 454 mM NaCl, 10 mM KCl, 10 mM $CaCl_2$, 27 mM $MgSO_4$, 26 mM $MgCl_2$, 2.4 mM $NaHCO_3$ and 0.8 mM NaBr. The half-diluted BAS culture media were used in the present study. The rotifers were precultured under a batch culture system at 25℃ using a cool-incubator model HCRCS2V150W-A1202 (Ikuta Industries, Tokyo, Japan) and subjected to a continuous feeding with commercially available concentrated algae *Nannochloropsis oculata* (Nikkai Center, Tokyo, Japan). Eggs deposited on the bottom in the precultures were collected and hatched out. Neonates were cultured under the same conditions as mentioned above in total darkness except during observation and subsequently divided into 2 groups: one for well-feeding (WF) as the control and the other for CR. CR was imposed by periodical food limiting at a 3 h/day feeding regimen. Culture media were changed daily at the beginning of the CR period. The rotifers in the CR group were transferred using a plankton net (50 μM mesh size) into a fresh medium without algae, whereas those in the WF group were into a fresh medium previously suspended with food algae. Samples were collected on day 2 using the plankton net and were washed 2 times with fresh BAS.

Total RNA extraction and poly (A)$^+$ RNA isolation

Total RNA extraction was performed using Isogen (Nippon Gene, Toyama, Japan) according to the manufacturer's protocols with a few modifications (Kaneko *et al*., 2002). Rotifers were collected and washed several times with fresh BAS during which food algae were filtered through the plankton net. Harvested rotifers (about 40,000 ind/200 ml) were homogenized with 1 ml Isogen in 1.5 ml tubes. The tubes were then swirled using a vortex and stored at room temperature for 5 min. An aliquot of 0.2 ml of chloroform (99.5%) was added into the tubes, which were subsequently shaken vigorously for 15 s and stored at room temperature for 2−3 min. After storage, the tubes were centrifuged at 16,000xg for 15 min at 4℃. The uppermost aqueous layers containing the extracted RNA were transferred into new tubes, added with 0.5 ml of isopropanol (99.5%), and stored at −20℃ overnight to precipitate RNA. The precipitated RNA was collected by centrifugation at 16,000xg for 30 min at 4℃. All aqueous phase was discarded and the precipitated RNA was washed with 1 ml ethanol (70%). The tubes were again centrifuged at 4,600xg for 10 min at 4℃. Alcohol was discarded and the tubes were dried briefly till the alcohol residues were completely evaporated. Finally, the RNA pellets were re-suspended into sterile distilled water. Poly (A)$^+$ RNA was isolated from the total RNA using Oligotex-dT30 (super) mRNA purification Kit (TaKaRa, Otsu, Japan) according to the manufacturer's instructions. RNA integrity was examined by using agarose gels containing 1% formaldehyde. The quantity and quality of RNA were determined by absorbance at A260 and at A260/280 using a DU® 530 Life Science UV/Vis spectrophotometer (Beckman Instruments, Inc., Fullerton, CA, USA).

Suppression subtractive hybridization (SSH) library

The present study focused on the genes that are differentially expressed in the rotifers under CR. Therefore, forward subtraction was performed for the CR group. cDNAs from the CR group were used as a tester, whereas cDNAs form the WF group were used as a driver. The testers were subtracted from the drivers (Fig. 1-2). SSH was performed using PCR-Select^{TM} cDNA Subtraction Kit (TaKaRa) with minor modifications. PCR amplification was conducted using a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA, USA). The optimized cycles for the primary and secondary PCRs were 27 and 15, respectively. The resulting subtracted cDNAs were ligated to the pGEM-T vectors using pGEM-T Vector Systems (Promega, Madison, WI, USA) and transformed into *Escherichia coli* strain JM109. White colonies were randomly picked up from the subtracted cDNA library and the presence of inserts was examined by agarose gel electrophoresis. The clones with inserts were grown in Luria–Bertani (LB) broth media containing ampicillin (200 μg ml^{-1}) overnight at 37℃ under shaking. Isolation of plasmid was accomplished using GenElute Plasmid Miniprep Kit (Sigma, St. Louis, MO, USA).

Sequencing and search for homologous sequences

The purified plasmid DNAs with inserts were subjected to PCR labeling using BigDye^{TM} Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and sequencing was performed using an ABI Prism 3100 Genetic Analyzer (Applied Biosystems). Adaptor and vector sequences flanking either side of partial sequences were detached using SeqEd v1.0.3 software (Applied Biosystems). Finally, modified expressed sequence tags (ESTs) were submitted to the NCBI database using the blastx and tblastn

programs in Basic Local Alignment Search Tool (BLAST) for searching any known gene counterparts with homologous sequences in the database.

Sequence annotation

Ontological annotation for differentially expressed genes was carried out by Gene Ontology (http://www.godatabase.org) followed by manual adjustment.

Results

Animal culture under calorie restriction

Rotifer cultures were performed using BAS. Neonates hatched out from the eggs were collected and cultured under the same conditions as mentioned before: a 3 h/day feeding regimen for the CR group, whereas the WF group was fed *ad libitum*. The WF group on day 2 showed observable reproduction and most individuals carried at least 2 eggs. However, the reproduction in the CR group was indistinct and very few individuals carried one egg. Thus, the numbers of eggs and individuals carrying eggs were remarkably different between the CR and WF groups, which were regarded as a visual parameter for distinguishing the effects of CR.

SSH and sequence homology

cDNA subtraction between the CR and WF groups was performed by SSH. As a forward subtraction, cDNAs from the CR group were used as a tester, whereas cDNAs from the WF group were used as a driver, and the driver cDNAs were subtracted from the tester cDNAs. After the secondary nested PCR, different band patterns were seen

between the subtracted and unsubtracted PCR products (Fig. 1-3). The bands corresponding to the molecular masses of 612, 495, 345, and 210 bp were predominantly observed in the subtracted PCR products. Subtracted PCR products were subcloned into the pGEM-T vectors and inserted clones were subjected to sequencing.

Randomly collected 163 clones containing inserts were sequenced and submitted to the NCBI database using the blastx and tblastn programs in BLAST. Among 163 ESTs submitted, 109 ESTs (67%) showed their homologous sequences, whereas other 54 ESTs (33%) did no significant similarity to known genes in the database. Sequence alignment of the ESTs with the same gene products was conducted using the ClustalW multiple sequence alignment program, yielding 38 different genes among 109 ESTs.

Sequence annotation

Gene ontology study showed one group of gene having unknown function (3%) and 6 functional groups of gene related to cellular structure, transport, and division (24%), DNA replication (11%), metabolism (36%), other functions (18%), transcription (5%), and RNA biosynthesis (3%) among 38 CR-induced differentially expressed genes revealed by SSH (Table 1-1 and Fig. 1-4).

The group related to cellular structure, transport, and division comprised 9 genes encoding lissencephaly-1 (*Lis1*), dynein heavy chain domain 3 (*Dnahc3*), beta 2 tubulin (*tub*), microtubule-associated protein EB 1 (*MT-EB1*), BRCA2 and CDKN1A-interacting protein isoform beta (*BCCIPβ*), calmodulin synthetic construct (*CaM64B*), spectrin beta chain (*Spnb1*), elongation factor Tu mitochondrial (*Ef-tu*), and proteasome regulatory particle, atpse-like protein (*rpt*). The group related to DNA

replication included 4 genes encoding CDT1 protein (*CDT1*), mismatch repair protein (*Msh6p*), DNA polymerase epsilon (*Pole*), and DNA polymerase sigma (*Pols*).

Fourteen genes were grouped under metabolism. Those were genes encoding stom protein (*stom*), galactose-4-epimerase (*Gale*), glycogen phosphorylase (*Glase*), β-glactosidase (*Glb*), 2-isopropylmalate synthase (*IPS2*), succinate dehydrogenase complex subunit D (*SDHD*), iron regulatory protein (*Irp*), peptidylglycine-hydroxylating monooxygenase (*Phm*), pancreatic amylase 2 (*Amy2*), alpha amylase (*αAmy*), phosphatidylserine/phosphatidylglycophosphate/cardiolipin synthase (*PS/PGp/Cl*), oligosaccharyl transferase STT3 subunit homolog (*stt3*), mitochondrial processing peptidase beta subunit (mtPP), and cytochrome *c* oxidase subunit II (*COX II*).

Seven genes were categorized into the group of other functions, and the group contained genes encoding tissue factor pathway inhibitor (*TFPI*), serine protease (*Ser*), multifunctional 14-3-3 family chaperone (*14-3-3*), serine/threonine phosphatase (*STPP*), serine/threonine protein kinase with TRP repeats (*PK-TRP*), transposase (*Tsase*), and mitochondrial 16S rRNA (*16S rRNA*).

Two genes encoding zinc finger protein (*Znf*) and EBF protein were in the same gene group that related to transcription. NOL1/NOP2/Sun domain family 2 protein encoding gene (*NSUN2*) retrieved its putative function in RNA biosynthesis. The gene encoding conserved hypothetical protein (*CHP*) was categorized as the gene with unknown functional properties.

Summary

cDNA subtraction was carried out between the rotifers of CR (fed 3 h/day) and WF (fed *ad libitum*) groups by SSH technique. To investigate the CR-induced differentially expressed genes in the rotifer *B. plicatilis*, cDNAs from the CR group were subtracted from those of the WF control group. Among 163 ESTs submitted to the NCBI database, 109 ESTs (67%) retrieved their homologous sequences from the database. Sequence assembly by ClustalW multiple sequence alignment program revealed 38 different genes among 109 ESTs. Subsequent Gene ontology study categorized 38 different genes as one functionally unclassified group and 6 functional groups. Six functional groups comprised genes with their putative functions in: cellular structure, transport, and division; DNA replication; metabolism; transcription; RNA biosynthesis; and other molecular functions.

Section 2: Validation of differentially expressed genes by semi-quantitative reverse transcription-PCR (RT-PCR)

Although SSH is a powerful technique for detecting the differentially expressed genes, which are needed for an organism responding to various environmental stimuli, it may sometimes yield non target genes that should not be detected under a particular conduction. Therefore, some additional studies are necessary to confirm the expression patterns of differentially expressed genes observed by SSH technique. In the present study, RT-PCR was used to further validate the mRNA levels of 38 differentially expressed genes. The mRNA levels of 38 genes were studied in the rotifers subjected to two different CR regimens, and the levels were compared between the rotifers of the CR group and its WF counterpart group.

Materials and methods

Feeding schedules for RT-PCR analyses

For RT-PCR experiments, rotifers were cultured and collected by the same procedures as mentioned in section 1, but fed with different feeding regimens. CR rotifers used in the first RT-PCR experiment were fed every 2 days, and those used in the second experiment were fed every 3 days, whereas their WF counterpart groups were fed *ad libitum* (Fig. 1-1).

cDNA preparations and RT-PCR

Total RNAs were extracted by the same procedures as mentioned in section 1

from 2 types of CR populations: one from CR group fed every 2 days and the other from CR group fed every 3 days as well as from their WF counterparts, and reverse-transcribed with oligo(dT) primers at 48℃ for 1.5 h using SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA). The reaction mixtures were subsequently heated at 70 ℃ for 15 min to inactivate the enzyme. RNAs complementary to cDNAs were removed by adding *E. coli* RNase H (Invitrogen) and the tubes were incubated at 37℃ for 15 min. The stock first strand cDNAs were diluted 50 times and the diluted first strand cDNA were used for PCR.

PCR was carried out using gene-specific primers constructed from the ESTs of 38 differentially expressed genes by Primer Express Software v2.0 (Applied Biosystems). Rotifer elongation factor-1α (EF-1α_ AB513493) was used as an internal standard and primers were designed from its cDNA fragment of 394 bp. The nucleotide sequences of primers are listed in Table 1-2. Thermal profiles of PCR were as follow: 95℃ for 5 min followed by 35 cycles at 94℃ for 30 s, at 50–56℃ for 30 s, and at 72℃ for 1 min. Electrophoretic photographing and documentation of PCR products were performed by using the Electrophoresis Documentation and Analysis System 120 (Eastman Kodak, New Haven, CT, USA). Although electrophoretic patterns of RT-PCR products were used to visually observe the accumulated mRNA levels of differentially expressed genes between the CR and WF groups, the electrophoretic documents were also used to determine the accumulated mRNA levels numerically. The mRNA levels of 38 differentially expressed genes were normalized to those of the internal standard, *EF-1α*. Molecular marker 5 (Nippon Gene) was used in all electrophoresis.

Results

Among 38 differentially expressed genes obtained by SSH, RT-PCR confirmed that 29 (76%) genes were up-regulated and 8 (21%) genes were down-regulated in the CR groups, whereas another one gene (3%) showed its unchangeable mRNA levels in both CR and WF groups. Their expressions were consistent in both RT-PCR analyses (Table 1-3 and Fig. 1-5).

Among genes related to cellular structure, transport and division, the mRNA levels of *Lis1* were clearly up-regulated in the CR groups. CR conferred 4.2- and 28.3-fold increases in the accumulated mRNA levels of *Lis1* in the first and second experiments, respectively. Up-regulation of *Dnahc3* was clearly observable (2.4-fold) in the second experiment. *BCCIPβ* showed its increased mRNA levels about 8.2-fold in the CR group of the first experiment. *CaM64B* showed its distinct up-regulated mRNA levels about 2.5-fold in the first RT-PCR.

Up-regulated mRNA levels of *Msh6p* reached 2.9- and 2.2-fold in the first and second experiments, respectively. The accumulated mRNA levels of *Pole* were 1.7-fold in the first and 2.4-fold in the second experiment. *CDT1* showed its up-regulation (4.1-fold) in the first experiment.

Although genes related to metabolism tended to be up-regulated in the CR group, most of their accumulated mRNA levels were not clearly distinct in the first and second experiments. However, *Glase* and *Glb* were clearly up-regulated in the second experiment, 1.5-fold and 1.9-fold, respectively. *IPS2* was clearly up-regulated (3.0-fold) by CR in the first experiment.

As for genes related to other functions, *14-3-3* was up-regulated by CR and its

up-regulation was distinct (1.5-fold) in the second experiment. *PK-TRP* was up-regulated 2.3-fold in the first experiment. The accumulated mRNA levels of *Tsase* were very distinct between the CR and WF groups. CR induced 20.9- and 6.6-fold up-regulation of *Tsase* in the first and second experiments, respectively. The mRNA levels of *16S rRNA* were up-regulated (1.5-fold) in the first experiment and its ESTs were accounted for 33 and apparently highest among 109 ESTs which showed homologous sequences.

Znf and *EBF* are considered to be those related to transcription. Their expression levels were higher in the CR group and they each showed 2.0-fold up-regulation in the first RT-PCR. *NSUN2* has a putative function in RNA biosythesis. Its mRNA expression was clearly up-regulated 2.5-fold in the first and 7.5-fold in the second experiments.

Summary

Since it is known that SSH yields false positive genes, 38 differentially expressed genes obtained by SSH were further validated for their expression levels under two different CR regimens by RT-PCR. Gene-specific primers were constructed from 38 target genes and used for two RT-PCR analyses. Rotifer elongation factor-1α (EF-1α) was used as an internal standard, and the mRNA levels of target genes were normalized to those of *EF-1α*. RT-PCR analyses verified that 29 and 8 genes were consistently up- and down-regulated in the CR groups, respectively, whereas another one gene showed unchanged mRNA levels in the CR and WF groups during both RT-PCR analyses.

Section 3: Discussion

SSH is a powerful technique to identify differentially expressed genes that are involved in physiobiological processes of organisms under a particular condition. In the present study, SSH was used to identify genes induced by CR in the rotifer *B. plicatilis*. Because SSH may yield false positives, further validation by RT-PCR was performed to reduce the false results from SSH. RT-PCR confirmed that 29 out of 38 different genes (76%) obtained by SSH were up-regulated in the CR group. No sequences homologous to any known plant genes in the database were observed among 38 different genes, suggesting no contamination of algal RNAs in this experiment. Annotated sequences were classified by Gene Ontology followed by manual adjustment, showing 6 functional groups for the 29 up-regulated genes (Table 1-1).

16S rRNA was most abundantly found (33 ESTs) among 109 ESTs encoding 38 different genes. It has been reported that *16S rRNA* was down-regulated in rat white adipose tissue after a high-fat diet (López *et al*., 2004), indicating diet-mediated *16S rRNA* expression and its possible roles in energy metabolism. Furthermore, the expression of *16S rRNA* decreases significantly with aging and this decrease correlates with the shape of the life span curve in *Drosophila* (Calleja *et al*., 1993). It has been reported that 16S rRNA expression depends on the cellular oxygen level (Ibrahim *et al*., 1998), and that the acute drop in 16S rRNA expression is attributable to oxidative stress (Crawford *et al*., 1997; Kowaltowski and Vercesi, 1999). It is possible that up-regulation of *16S rRNA* in the present study retards aging and hereby the rotifer gains a longer life span under CR. Among genes related to cellular structure, transport and division, the number of EST encoding *Dnahc3* was 17, the second most abundant transcript. Dynein

motor protein has several roles, in combination with other molecules, in cellular activities. In *Drosophila*, dynein is required during germline cell divisions and oocyte differentiation (McGrail and Hays 1997). The dynein heavy chain gene is differentially expressed during development with the highest levels of transcripts in ovaries and embryos (Li *et al*., 1994). Dynein localization along the oocyte cortex in wild-type *Drosophila* egg chambers is dependent on *Drosophila* Lis1, DLis1 (Swan *et al*., 1999). It has been speculated that a membrane-associated protein, spectrin, is required for proper localization of DLis1 to the oocyte cortex in the *Drosophil*a ovary (Swan *et al*., 1999). Lis1 interacts physically with β-spectrin in vitro (Wang *et al*., 1995). In the present study, the gene encoding β-spectrin was also observed among 38 different genes, but RT-PCR analysis showed its expression down-regulated by CR. Spectrin has been suggested to function in oocyte growth and differentiation or in the organization of a polarize microtubule based RNA transport system as it does in *Drosophila* (Deng and Lin, 1997). Based on these findings and the present results, up-regulation of *Dnahc3* and *Lis1* and the concomitant down-regulation of the gene encoding β-spectrin are probably attributable to reproductive suppression of the rotifer under CR. CaM64B is a synthetic construct of calmodulin (CaM), a calcium-binding protein. Calcium metabolism is an important part of oocyte development, and calcium within cells requires a binding protein as a mediator such as CaM (reviewed in Carafoli, 1987). It has been suggested that CaM has a crucial role in oogenesis (Cicirelli and Smith, 1986). CaM has been reported to associate with microtubule and it may influence microtubule assembly (Deery *et al.*, 1984). Therefore, up-regulated *CaM64B* might exert its activities together with *Lis1*, *Dnahc3* and *Spnb1* on oocyte growth. BCCIPβ is an isoform of BCCIP, a BRCA2 and CDKN1A (p21 or $p21^{Waf1/Cip1}$) interacting protein.

BCCIPβ interacts with p21 *in vivo*, inhibits cell growth, and delays progression of G1 to S phase (Meng *et al*., 2004). It has been reported that CR decreases the rate of cell division as well as the total number of dividing cells in rat colonic mucosa (Albanes *et al*., 1990). In the present study, *BCCIPβ* expression was up-regulated by CR. Dietary restriction retards the rate of organ-specific nuclear division in the rotifer *A. brightwelli* (Verdone-Smith and Enesco, 1982). Cell division in the rotifer is known to occur only in their eggs and CR suppresses reproduction (Egami, 1972). Taken together, up-regulation of *BCCIP* may regulate the metabolic shift of rotifers from reproduction to body maintenance under CR.

In addition to its roles in cell cycle delays, BCCIP has an essential role in the maintenance of genomic integrity, especially in chromosome stability (Meng *et al.*, 2007). BCCIP functions in genome stability through its direct interaction with homologous recombinational repair (HRR) protein, BRCA2 (Lu *et al*., 2005), indicating BCCIP participates in DNA repair. Mismatches result from DNA replication errors and genetic recombination, and DNA damages are fixed in the genome if uncorrected (Crouse, 1996; reviewed in Kolodner, 1996; in Modrich and Lahue, 1996; in Modrich, 1997). Mismatches are corrected by mismatch repair proteins, Msh2p and Msh6p (Alani, 1996; Iaccarino *et al*., 1996). Msh6p has been implicated in somatic DNA stability (Tijsterman et al., 2002). In this study *Msh6p* was up-regulated by CR, suggesting that CR induces the expression of genes involved in DNA repairs and their expression is attributable to genome stability. In this study, *Pole*, *Pols*, and *CDT1* were up-regulated by CR. In *Saccharomyces cerevisiae*, Pols is necessary to stimulate DNA polymerase activity of Pole holoenzyme (Edwards *et al*., 2003) and this activity is also required for rapid and efficient chromosomal DNA replication in *Xenopus*, a higher eukaryote

(Shikata *et al*., 2006). The yeast TRFs, now designated Pols, are essential for completion of S phase, for DNA repair, and for efficient sister chromatid cohesion (Castano *et al.*, 1996; Aravind and Koonin, 1999; Wang *et al.*, 2000; Burgers *et al.*, 2001). Pole participates in DNA repair (Wang *et al.*, 1993), and cooperates with other polymerases and mismatch repair to limit spontaneous mutation (Albertson *et al.*, 2009). Pole in *S. cerevisiae* localizes and functions at the replication forks (Hiraga *et al*., 2005) and its expression peaks at G1/S (reviewed in Sugino, 1995). It has been suggested that Pole in yeast is essential for cell viability (Morrison *et al.*, 1990), and also required for S-phase progression (Feng and D'Urso, 2001; Ohya *et al.*, 2002). However, the precise roles of Pole in DNA synthesis remain obscure and its activities in cell viability are still debatable (Dua *et al*., 1999; Kesti *et al.*, 1999; Feng and D'Urso, 2001). CDT1, like Pole, is also specifically recruited to chromatin during G1 and S phase (reviewed in Bell and Dutta, 2002). However, Pole and CDT1 are antagonistic to each other in regard to DNA replication processes; Pole is required for the efficient elongation of nascent DNA (Waga *et al.*, 2001), whereas increased CDT1 activity inhibits the elongation of nascent strands in *Xenopus* egg extracts (Tsuyama *et al.*, 2009). It has been suggested that controlling CDT1 is important for genome stability, and CDT1 has been reported to have an oncogenic potential when etopically expressed in cells (Arentson *et al.*, 2002). On the other hand, CDT1 activity in DNA replication is inhibited by addition of supplementary CDT1 itself (Tsuyama *et al.*, 2009). Therefore, an up-regulation of *CDT1* seems to be antagonistic not only to *Pole* but also to *CDT1* itself as well. These might be possible factors that suppress DNA replication in the CR rotifers, with the result that cell cycle progression is inhibited. Regarding cell cycle activities, iron is an essential element for cell growth and development, contributing to DNA synthesis and

regulating the G_1- to S-phase transition. Intracellular iron metabolism is regulated by Irp. The regulatory link between iron metabolism and the cell cycle has been reported (Sanchez *et al.*, 2006). Irp translationally regulates citric acid cycle enzymes via iron-responsive elements (Gray *et al.*, 1996), suggesting possible roles of Irp in metabolism through mitochondrial biogenesis. The binding of Irp to 5′ untranslated region (5′-UTR) of iron responsive element (IRE) inhibits translation initiation of the target mRNA, whereas the binding to 3′-UTR of IRE stabilizes the target mRNA (Muckenthaler *et al*., 1998; reviewed in Schneider and Leibold, 2000; reviewed in Eisenstein and Ross, 2003). Regarding these studies, *Irp* up-regulated under CR and CR-like stationary phase might participate in a regulatory link between energy and iron metabolism and regulate the translation of other mRNAs transcribed in the CR rotifers. *Irp* was up-regulated by CR in the present study. In *D. melanogaster*, Irp binds to an IRE in the 5′-UTR of mRNA encoding succinate dehydrogenase (SDH) subunit B, SDHB (Gray *et al*., 1996). SDH subunit D (SDHD, a mitochondrial associated enzyme) was up-regulated by CR in the present study. Studies in tumor cells indicated that *SDHD* mutation resulted in cellular hypoxia (van Nederveen *et al.*, 2003). According to these studies, the up-regulation of *SDHD* might protect cellular oxidative damage and oxidative stress, especially in mitochondria, of the rotifers under a limited food condition. Thus, the CR rotifers would attain a longer life span. TFPI is the major physiologic inhibitor of tissue factor (TF) pathway. TFPI induces the expression of *GADD45B* participating in growth arrest and DNA damage (Shirotani-Ikejima *et al*., 2002). GADD45 arrests the cell cycle and is involved in DNA nucleotide excision repair (Kastan *et al*., 1992; reviewed in Levine, 1997). TFPI has been shown to inhibit cell proliferation through mechanisms independent of its anti-coagulant activity

(Hembrough *et al.*, 2001). Recently, TFPI has been known to have an antibacterial activity (Schirm *et al.*, 2009). *TFPI* was up-regulated by CR in the present study and its up-regulation might contribute to cell cycle arrest at G1/S phase and also to the fitness of CR organisms. NSUN2 is an RNA methyltransferase. NSUN2 has high sequence homology to mammalian Misu protein, which contains SUN domain. It has been reported that *Misu* expression is highest in S phase (Frye and Watt, 2006). Because rotifers are eutelic (animals with about 1,000 total cells in the adult form) and their cell division only occurs in their eggs (Egami, 1972), up-regulation of cell phase-related genes by CR is possibly due to other functions. For example, TFPI is a physiological inhibitor of coagulation and has biological functions of anticoagulation and anti-inflammation (Bai *et al*., 2005). There is evidence, which suggests that inhibition of inflammatory response and age-related inflammation by CR are key mechanisms involved in CR-induced beneficial effects (reviewed in Chung *et al*., 2001, 2006; Jolly *et al*., 2001). Meanwhile, up-regulation of *NSUN2* might be related to DNA methylation, since CR increases genomic methylation of oncogenes like ras DNA in the cells taken from CR rats (reviewed in Hass *et al*., 1993) and the management of DNA-related processes that are signaled by the diet is the methylation of DNA (Adams and Burdon, 1985; reviewed in Hergersberg, 1991).

CR not only functions at cellular and molecular levels, but also has many effects on metabolism of various animals. Lowered plasma glucose content consequent to a variety of CR regimens has been demonstrated in mouse, rat, and non-human primates of different ages (Masoro *et al*., 1992; Harris *et al*., 1994; Kemnitz *et al*., 1994; Cefalu *et al.,* 1995). The stom protein, also called stomatin, is a 32-kDa integral membrane protein. Stomatin associates with glucose transporter molecule 1 (Glut1) in

the plasma membrane and its overexpression results in a depression in the basal rate of glucose transport (Zhang *et al.*, 1999), indicating that stomatin participates in metabolism through the association with glucose transporter proteins. Therefore, up-regulation of *stom* under both CR might directly regulate the glucose uptake of cells of the rotifers under a limited food condition. On the other hand, stomatin has two other possible biological functions: first, regulation of ion channel function; second, acting as a cytoskeletal anchor (reviewed in Stewart, 1997). It has been reported that stomatin had a finite binding affinity for the spectrin-actin cytoskeleton, and also associated with microtubules (Umlauf *et al.*, 2004). Based on these studies, *stom* in the present study might functionally interrelate with *Lis1*, *Dnahc3*, *Spnb1* and *CaM64B* in cellular localization processes, especially during oogenesis. *Glase* and *Glb* were up-regulated by CR in this study. Glycogen is degraded for metabolic use by Glase, liberating glucose units from the liver cells into bloodstream. Glb (also called lactase) is commonly used to cleave lactose into galactose and glucose. Lactase activity is consistently higher in dietary restricted animals than their counterparts fed *ad libitum* (Maier *et al*., 2007). The up-regulation of these genes is likely to regulate the body glucose levels required for maintaining important metabolic processes under CR. The up-regulation of *IPS2* was remarkable. Enzyme IPS2 in *S. cerevisiae* catalyzes leucine biosynthesis (Ryan *et al*., 1973). Leucine is an essential amino acid and also a potent activator of serine/threonine kinase involved in many cellular processes, including protein synthesis, cell growth, and metabolism (reviewed in Inoki *et al*., 2005; Cota *et al*., 2006). The mammalian target of rapamycin protein (mTOR) is a highly conserved serine/threonine kinase. It has been reported that leucine exerts its activity on hypothalamic mTOR signaling in food intake and energy balance regulation in a mammalian model (Cota *et al.*, 2006). In this study,

PK-TRP was also up-regulated as the case of *IPS2*.

14-3-3 is a ubiquitous family of highly conserved eukaryotic proteins from fungi to human and plants with several molecular and cellular functions. The members of the 14-3-3 family mediate interactions between diverse components having different biological activities. 14-3-3 proteins have been implicated in the regulation of cell cycle (Stoica *et al*., 2006). 14-3-3 regulates life span in *C. elegans* by both DAF-16 (a member of FOXO/forkhead transcription factor family)-dependent and -independent mechanisms (Berdichevsky *et al*., 2006; Wang *et al*., 2006; Araiz *et al*., 2008). Therefore, life span extension in *B. plicatilis* under CR is attributable to the up-regulation of *14-3-3*. Because of its diverse biological functions, the up-regulation of *14-3-3* would be important for various biological processes under CR.

Tsase confers translocation of transposable element (TE) in the genome. It has been observed that TEs are differentially expressed in black tiger shrimp *Penaeus monodon* exposed to a range of environmental stressors (de la Vega *et al*., 2007). In fish, the expression of *Tsase* is induced by external stimuli such as toxin, stress, and bacterial antigens (Krasnov *et al*., 2005). Based on previous findings and the present results, *Tsase* expression seems necessary for responding to various environmental stressors.

EBF is a transcription factor known to be responsible for the development of B-lymphocytes. *Collier* (*col*, the *Dorsophila* ortholog of the vertebrate gene encoding EBF) has been implicated in developing lamellocytes, which function in cellular immune response to parasitization in *Drosophila* (Crozatier *et al*., 2004). Therefore, the up-regulation of *EBF* might protect the rotifer from various potential diseases under CR.

The majority of up-regulated genes by CR in this study show similar expression patterns in other CR animals. Genes related to cell growth and maintenance

(Pletcher *et al*., 2002), energy metabolism (Lee *et al*., 1999; Sreekumar *et al*., 2002; reviewed in Park and Prolla 2005), and DNA synthesis (reviewed in Park and Prolla 2005) are generally up-regulated by CR.

CHAPTER 2

Comparison in expression levels of selected genes between WF and CR groups containing individuals of various ages as well as between populations at exponential and stationary phases

Seventeen differentially expressed genes were selected for quantitative real-time RT-PCR (qRT-PCR) analyses. Genes were selected according to their putative functions and their nucleotide sequence lengths possible for designing qRT-PCR primers. The selected genes covered 4 functional groups: (1) DNA replication; (2) cellular structure, transport, and division; (3) metabolism; and (4) other functions. Group 1 comprised *CDT1*, *Msh6p*, *Pole* and *Pols*. Group 2 consisted of *Lis1*, *Dnahc3*, *BCCIPβ*, *CaM64B* and *Spnb1*. Group 3 contained *stom*, *SDHD*, *Irp* and *Amy2*. *TFPI*, *Tsase*, *16S rRNA* and *14-3-3* were categorized as the members of group 4. Among the selected genes, all genes except *Spnb1* and *Amy2* were further confirmed to be up-regulated in the CR rotifers by RT-PCR analyses. Although *Spnb1* and *Amy2* were obtained from the subtracted cDNA library of the CR rotifers, subsequent RT-PCR analyses showed that the two genes were down-regulated in the CR rotifers.

The expression patterns of the selected genes were studied under 2 experimental designs by qRT-PCR analyses. In section 1, the mRNA levels of the selected genes were investigated in the CR and WF rotifers of various age compositions. The mRNA levels were studies on eight consecutive culture days and the levels were compared between the CR and WF groups.

In section 2, the mRNA levels of the selected genes were studied in the

exponential and stationary phases of a population growth. As the CR-like phenomena might occur during the stationary phase of a population growth, the mRNA levels of the selected genes were studied in the CR-like stationary and WF-like exponential phases. The relative mRNA levels were compared between the exponential and stationary phases.

Finally, the expression patterns of the selected genes under CR and CR-like stationary phase were compared.

Section 1: Expression levels in the WF and CR populations of various ages

CR is the most effective intervention known to extend life span in a variety of species (Weindruch and Walford, 1988), although there have been debates on the benefits of CR in some laboratory animals. The beneficial effects of CR can be observed not only when initiated at a young age, but also in adulthood. Animals under a CR regimen maintain most physiological functions in a youthful state at more advanced ages, but the effects of CR can vary with the age of animals at which CR is applied. Thus, a population of various age compositions under CR might consist of various individuals which have different metabolic properties in response to CR. The metabolic processes of every individual are governed at the molecular levels by the expression of suitable genes, and the expression might be varied among individuals of various ages. These different gene expressions among individuals result in a metabolically non-uniform population under a particular condition and lead to the population that fluctuates in size. In this section, the mRNA expression patterns of the selected genes

were studied in the CR and WF rotifer populations of various age compositions.

Material and Methods

Animal culture and sample preparation

The same species, culture system, culture medium, culture temperature, and food described in Chapter 1 were used in this experiment. The rotifers were randomly collected from the stocks and divided into CR and WF groups. Six experimental cultures, 3 each for the CR and WF groups, were begun with about 100 rotifers/ml randomly collected from the stock culture and reared in 100 ml beakers for eight consecutive days. The CR group was fed 3 h/day as in the cDNA subtraction experiment, whereas the WF group was fed *ad libitum* (Fig. 2-1). The culture media in both the CR and WF groups were changed every day after feeding for three hours in the CR groups.

The samples were collected on eight consecutive culture days. Thirty individuals were randomly sampled from every experimental culture of the CR and WF groups. Total RNA extraction and cDNA synthesis were carried out by the same procedures described in Chapter1. The stock of first strand cDNAs were diluted 100 times and the diluted first strand cDNAs were used in qRT-PCR analyses.

Quantitative real-time reverse transcription-PCR (qRT-PCR)

Quantitative real-time RT-PCR using SYBR® *Premix Ex Taq*™ (TaKaRa) was performed on 7300 Real Time PCR system (Applied Biosystems). Primers for real-time RT-PCR (Table 2-1) were designed using Primer Express Software v2.0 (Applied Biosystems). A 20 μl of reaction mixture comprised 10 μl of 2×SYBR *Premix*

Ex Taq, 0.3 μl of 50×Rox Reference Dye II, 1 μl of primer mix (20 μM), 1 μl of 100-time diluted template cDNA, and 7.7 μl of H_2O. A two-step absolute quantification RT-PCR method was applied to PCR under default thermal cycling conditions, and dissociation temperatures were also determined. Relative mRNA levels of the target genes were determined by a comparative Ct method with reference to that of *EF-1α* as the internal control.

Data analysis

Student's *t*-test (two-tailed) was employed for the statistical analyses. Data were shown in means with standard errors.

Results

The mRNA levels of the genes related to DNA replication were up-regulated in the CR group and their up-regulation were found on all CR days from day 2 to 8 (Fig. 2-2). The mRNA levels of *Msh6p* were significantly up-regulated in the CR group from day 2 to day 7 (Fig. 2-2A). *Pole* was significantly up-regulated in the CR group on all CR periods from day 2 to 8 (Fig. 2-2B). *CDT1* showed it up-regulation on all CR days, but the significance level was detected only on days 4, 5, 7 and 8 (Fig. 2-2C). *Pols*, like *Pole*, was also significantly up-regulated in the CR group on all CR periods from day 2 to 8 (Fig. 2-2D).

Genes with their putative functions in cellular structure, transport and division were up-regulated on most of CR days. The mRNA levels of *BCCIPβ* in the CR and WF groups were nearly the same on day 8, but the levels on other days were higher in the

CR group. However, the significance level was found only on days 4, 5 and 6 (Fig. 2-2E). *Lis1* showed no different mRNA levels between the CR and WF groups on days 6 and 8. The significant up-regulation of *Lis 1* was found on days 3, 4 and 5 (Fig. 2-2F). *Dnahc3* showed its significant up-regulation in the CR group on all CR days except on day 6 (Fig. 2-2G). The mRNA levels of *CaM64B* were significantly up-regulated in the CR group on all CR days (Fig. 2-2H). *Spnb1* showed a two-edged expression pattern; the mRNA levels of *Spnb1* were significantly up-regulated in both the CR and WF groups (Fig. 2-2I). In the CR group, the significant up-regulation of *Spnb1* was observed on days 7 and 8, whereas in the WF group, on days 5 and 6.

Among the genes related to metabolism, *stom* was significantly up-regulated in the CR group on all CR days (Fig. 2-2J). *SDHD* also showed its up-regulation in the CR group on all CR days (Fig. 2-2K). The up-regulation of *Irp* in the CR group was significant on days 3, 4, 5 and 7 (Fig. 2-2L). *Amy2* showed up- and down-regulation in both CR and WF groups (Fig. 2-2M). In the CR group, *Amy2* was significantly up-regulated on days 3, 4, 5 and 7. In the WF group, *Amy2* was significantly up-regulated on days 2 and 7.

In the group of genes related to other functions, *TFPI*, *Tsase* and *16S rRNA* showed their significant up-regulation in the CR group on all CR days (Fig. 2-2N, O, P). The mRNA level of *14-3-3* was up-regulated in the CR group on days 2, 3, 4 and 8 (Fig. 2-2Q).

The expression in both the CR and WF groups was assumed to be up-regulated if at least $p<0.05$ level of significance was found on 2 consecutive culture days. Therefore, all selected genes except *Spnb1* were up-regulated in the CR rotifer population of various ages.

Summary

Quantitative real-time RT-PCR analysis showed that most of the selected genes had high possibilities of being up-regulated in the CR group. In the group of genes concerning DNA replication, all showed high tendencies to be up-regulated in the CR group and up-regulation of *Pole* and *Pols* in the CR group was significant on all consecutive CR days.

In the group of cellular structure, transport and division, all genes except *Spnb1* showed higher mRNA levels in the CR group and significant up-regulation of *CaM64B* was observed on all consecutive CR days. *Spnb1* showed the significant up- and down-regulation in both CR and WF groups.

Among the genes related to metabolism, all genes except *Amy2* showed significant up-regulation in the CR group, whereas *Amy2* had significant up- and down-regulation in both CR and WF groups.

In the group of genes related to other functions, all genes had significant up-regulation in the CR group. Significance levels of *TFPI*, *Tsase* and *16S rRNA* were observed on all consecutive CR days.

Among 17 selected genes, all genes except *Spnb1* were assumed to be up-regulated in the CR group of various ages because their significant up-regulation in the CR group were observed on two consecutive CR days.

Section 2: Expression levels at the exponential and stationary phases of a population growth

Generally, CR methods differ in the type of food that is limiting; the time at which CR is applied; and the type of medium in which CR is induced. These differences would vary the benefits caused by CR even among individuals of the same species. CR in fact is a laboratory-replicated environmental condition under which animals have less chance to access to the amount of food per individual. In nature, animals encounter such kind of CR-like conduction in the stationary phase of a population growth, where a daily food resource is limited for growing population. Studies on the behavioral and metabolic changes of animals under CR and CR-like conductions would give more reliable information necessary for managing the food-related problems occurring in both natural and laboratory populations. At the molecular level, knowledge about the genes and their expression patterns under certain environmental stimuli is prerequisite to the management of organismal metabolic processes, which response to the stimuli.

In this section, the mRNA levels of the selected genes were examined in the exponential and stationary phases of population growth by qRT-PCR. The mRNA levels of the target genes were normalized to that of the internal standard, *EF-1α*, and the relative mRNA levels were compared between the CR-like stationary and WF-like exponential phases.

Materials and methods

Animal culture and sampling

The same species, culture system, culture medium, culture temperature, and food described in Chapter 1 were used in this experiment. Ten experimental cultures, 5 each for the two phases, were commenced with adult 100 rotifers without eggs and the rotifers were reared in 100 ml beakers. The daily food amounts were kept at a constant level among the cultures. The rotifers in each culture were fed every day with 1 ml of algae (approximately 7×10^6 cells/ml). Everyday at the time of feeding, 95 ml of old media containing the rotifers were transferred into the new beakers already filled with 1 ml of food algae plus 4 ml of BAS. The bottom parts of 5 ml volumes littered with the old food and animals' debris were discarded. After changing into new beakers, 1-ml culture media were collected from every culture for counting the number of individuals. The rotifers were subsequently fixed into paraformaldehyde (PFA) with a final concentration of 2%, and the numbers were counted after fixation daily.

The rotifers from 5 beakers of a 100 ml volume (17,000 individuals/100 ml) were sampled for the exponential phase on day 8, whereas those from the other 5 beakers of a 100 ml volume (60,000 individuals/100 ml) were collected for the stationary phase on day 15 (Fig. 2-3).

Total RNA extraction and cDNA synthesis

Total RNA extraction and cDNA synthesis were carried out as the same procedures described in Chapter1. The stock of first strand cDNAs was diluted 100 times and the diluted first strand cDNAs were used in qRT-PCR analyses.

Quantitative real-time RT-PCR (qRT-PCR) and data analysis

Quantitative real-time RT-PCRs and data analyses were performed as the same procedure described in Section 1 of this Chapter. The number of samples examined was 5.

Results

Fourteen selected genes were significantly up-regulated in the stationary phase (Fig. 2-4). Among four genes related to DNA replication, *Msh6p* and *Pole* were significantly up-regulated in the stationary phase at $p<0.01$ and $p<0.001$, respectively (Fig. 2-4A, B). In contrast to the expression of *Msh6p* and *Pole*, the other 2 DNA replication-related genes, *CDT1* and *Pols*, showed their up-regulation in the exponential phase (Fig. 2-4C, D). However, the level of significance at $p<0.01$ was observed only in *CDT1* expression.

In the group of genes related to cellular structure, transport and division, four genes, *BCCIPβ*, *Lis1*, *Dnahc3* and *CaM64B*, were significantly up-regulated in the stationary phase (Fig. 2-4E, F, G, H). The significance level for *BCCIP β* was $p<0.01$ and for *Lis1*, *Dnahc3* and *CaM64B* was $p<0.001$. The other one gene, *Spnb1*, in the same group was up-regulated in the exponential phase, but its expression was not statistically significant (Fig. 2-4I).

All metabolism-related genes, *stom*, *SDHD*, *Irp* and *Amy2*, were significantly up-regulated in the stationary phase at $p<0.001$ level (Fig. 2-4J, K, L, M).

All genes related to other functions, *TFPI*, *Tsase*, *16S rRNA* and *14-3-3*, were significantly up-regulated in the stationary phase at $p<0.001$ level (Fig. 2-4N, O, P, Q).

Summary

As expected, 14 out of 17 selected genes (82%) were significantly up-regulated in the stationary phase. The up-regulated genes were *Msh6p*, *Pole*, *BCCIPβ*, *Lis1*, *Dnahc3*, *CaM64B*, *Amy2*, *stom*, *SDHD*, *Irp*, *TFPI*, *Tsase*, *16S rRNA* and *14-3-3*. The other 3 genes, *CDT1*, *Pols* and *Spnb1* (18%) showed down-regulation in the stationary phase, but the significance level was found only with *CDT1*.

Section 3: Discussion

Among 38 differentially expressed genes previously obtained by SSH, 17 genes were selected to examine their expression under two experimental designs. Fifteen out of 17 selected genes had been already confirmed by RT-PCR for their up-regulation in the CR group, whereas other 2 genes, *Spnb1* and *Amy2*, had shown their down-regulation in the CR group.

In this Chapter, the expressions of 17 selected genes were examined at several sampling points over 8 culture days by qRT-PCR in the CR and WF rotifer populations of various ages. Subsequently, their expressions were also examined in CR-like stationary phase of population growth. If the expressions of the selected genes were affected by a limited food condition, then their expressions should be similar during CR and CR-like stationary phase.

As expected, 14 genes *Msh6p*, *Pole*, *BCCIPβ*, *Lis1*, *Dnahc3*, *CaM64B*, *Amy2*, *stom*, *SDHD*, *Irp*, *TFPI*, *Tsase*, *16S rRNA* and *14-3-3* were consistently and significantly up-regulated in both CR and CR-like stationary phase, indicating their expressions were

induced by a limited food condition. In Chapter 1, RT-PCR validated *Amy2* was down-regulated in the CR groups. However, subsequent qRT-PCR analyses showed that the mRNA level of *Amy2* was significantly up-regulated under CR and CR-like stationary phase. As being a digestive enzyme, the up-regulation of *Amy2* in the rotifers under limited food conditions is interesting. In rotifer live food production, knowledge of digestive physiology and of capacity of response to food composition and feeding period would be important for managing the population of rotifer efficiently.

Other 3 genes *CDT1*, *Pols* and *Spnb1* showed their expressions down-regulated in the CR-like stationary phase, even though *CDT1* and *Pols* were up-regulated under CR. In Chapter 1, RT-PCR confirmed that *Spnb1* was down-regulated in the CR groups. *Spnb1* showed ambiguous expression patterns in the CR experiment; *Spnb1* had significant up-regulation in both the CR and WF rotifers. *Spnb1* expression seemed not to be CR-specific, as its expression level was also higher in the exponential phase of population growth. Therefore, based on the qRT-PCR analyses done under two different experimental designs, the expressions of these three genes were not likely to be influenced by a limited food condition. Because the numbers of samples and sampling points taken for qRT-PCR analyses were large enough to carry out statistical tests, qRT-PCR results gave more reliable data for the selected genes. However, the previous results obtained by SSH and RT-PCR were indispensable for the present study. In fact, qRT-PCR analyses further proved their validity.

The discrepancies, between the expression patterns obtained by SSH and RT-PCR, might be due to the differences in the magnitude of CR and in the number and age of individuals among the samples, and are most probably due to the transient expression and diverse functions of the differentially expressed genes under CR.

CHAPTER 3

Comparison in DNA synthesis and expression levels of selected genes between CR and WF groups of the same ages

Since rotifers are primarily eutelic: each adult organ contains a constant, species-specific number of cells (or nuclei, in syncytial tissues), the only organs where cell division might be influenced by dietary restriction are those where cell division continues into adult life: the gastric glands which aid in digestion and the vitellarium which provides nutrients to the developing embryo (Jones and Gilbert, 1977). The vitellarium is connected to both immature and maturing oocytes by broad cytoplasmic channels (Birky *et al,.* 1967) and each oocyte is joined to the vitellarium through a cytoplasmic bridge (Bentfeld, 1971a). The oocyte is relatively inert during the entire growth and its cytoplasmic volume appears to be provided by the vitellarium through the connecting bridge (Bentfeld, 1971b).

It has been proposed that extra copies of the vitellarium genome are required to support rapid synthesis of the cytoplasm which is supplied to the maturing oocyte (Birky *et al,.* 1967). In the vitellarium, nuclei are presumably becoming polyploid. The nuclear division in the gastric glands and vitellarium is slowed down in dietrary-restricted rotifer *A. brightwelli*. Thus, the rotifer increases both the mean life span and the length of the reproductive period compared to its well-fed counterpart (Verdone-Smith and Enesco, 1982). Because it has been suggested that reproduction, specifically oocyte growth, in rotifer relies on the extra copies of the vitellarium genome (Birky *et al.*, 1967), it is likely that a longer life span in CR rotifers may be due

to the reproductive suppression by lowering the nuclear division rate in the vitellarium. Then, CR rotifers delay senescence and lengthens reproductive period throughout their life span.

The reason why CR increases life span in numerous organisms is largely unknown. It can be suggested that rotifers that attain a longer life span may have a reduced rate of living where reproduction may be most affected because CR-induced reproductive suppression is common to various animals. As the vitellarium is the largest and most conspicuous part of the reproductive system of rotifers, CR may directly exerts its effects on vitellarium metabolism where the nuclear division is slowed down by CR.

In the previous Chapters, genes related to DNA replication, cell cycle, metabolism and other functions were observed in the CR subtracted cDNA library. Subsequent studies by RT-PCR and qRT-PCR fortified that most of the genes had high potential for being up-regulated by CR. Therefore, DNA synthesis as well as the expression patterns of the selected genes was further examined in the CR and WF rotifer populations of the same ages.

Section 1: BrdU labeling for detecting DNA synthesis

Since Reichard and Estborn (1951) introduced a method to trace DNA synthesis by incorporation of radioactively labeled thymidine ([^{3}H]-T), the method has been used successfully in the analytical cell biology. Nevertheless, this method has several disadvantages such as technical difficulties associated with the detection of [^{3}H]-T. These impediments led to the development of new nonradioactive nitrogen base analogs such as 5-bromo-2′-deoxyuridine (BrdU).

BrdU is an analogue of thymidine and incorporated into newly synthesized DNAs of proliferative cells and the incorporated BrdU then can be detected immunocytochemically by a specific anti-BrdU monoclonal antibody (Gratzner, 1982). The development of highly specific antibodies to BrdU (Gratzner, 1982; Gonchoroff et al., 1986; Magaud et la., 1988) made it the most popular marker currently used to detect cell proliferation. BrdU has been reported to be useful for detecting the DNA synthesis as well as the cell proliferation activity in aquatic animals (Plickert and Kroiher, 1988; Zaldibar *et al*., 2008).

In this section, DNA syntheses in both the CR and WF rotifer populations of the same ages were studied by BrdU labeling.

Materials and methods

Animal culture and sampling

For the experimental cultures, the same species, culture system, culture medium, culture temperature, and food described in Chapter 1 were used in this

experiment. Eggs shed onto the bottom of the stock cultures were collected and hatched out. After 3 hour-post-hatching (hph), neonates presumably of the same ages were transferred into new media and fed with algae (Fig. 3-1). After the first 3-hour- feeding period, the rotifers were divided into 2 groups: one for the CR group and the other for the WF group. Three experimental cultures each for the two groups were raised. Each culture was begun with about 400 rotifers which were reared in 12-well cell culture plates with the culture volume of 5 ml. The CR group was fed 3 h/day, whereas the WF one was fed *ad libitum*.

Sampling in both the CR and WF groups was carried out every three hours from 3 hph to 39 hph stages. Twenty individuals were collected from each culture for both the CR and WF groups, and totally 60 individuals for one sampling point were collected for each group.

BrdU labeling and signal detection

The rotifers were incorporated with BrdU (Wako, Osaka, Japan) in the artificial sea water already dissolved with a final concentration of 5 mM BrdU. The infusion of BrdU into the rotifers was performed at room temperature for 15 min (Fig. 3-2). For the negative control, the rotifers were not incorporated with BrdU and directly subjected to fixation. The rotifers were fixed in serially concentrated (0.05, 0.1, 0.2, 0.5, 1, 2, and 3 %) paraformaldehyde/phosphate-buffered saline (PFA/PBS, pH 7.0) at room temperature for 5 min each. The samples were subsequently transferred and fixed in 4% PFA/PBS (pH 7.0) at 4℃ overnight. In the following day, the samples were washed 2 times in 0.25% Triton/PBS (pH 7.2) at room temperature for 30 min each. An additional wash in 0.4 M glycine/PBS (pH 7.2) was performed 2 times at room temperature for 30

min each. DNA was denatured in 2 N HCl/PBS (pH 7.2) at room temperature for 1 h. The samples were washed 2 times in 0.25% Triton/PBS (pH 7.2) at room temperature for 30 min each. Prior to the anti-BrdU incubation, anti-BrdU (Sigma-Aldrich) was diluted 1:1000 into a 10% goat serum albumin in 0.25% Triton/PBS (pH 7.2). Anti-BrdU incubation was done at 4℃ overnight. Next day, the samples were washed 2 times in 0.25% Triton/PBS (pH 7.2) at room temperature for 30 min each. Before the secondary antibody incubation, the antibody (Goat-anti-mouse IgG conjugated with an Alexa fluor 555 (Invitrogen) was diluted 1:500 into a 10% goat serum albumin in 0.25% Triton/PBS (pH 7.2). The secondary antibody incubation was done at room temperature for 2 h. The samples were washed 3 times with 0.25% Triton/PBS (pH 7.2) at room temperature for 10 min each. The samples were then counter-stained with a 1 μg/ml DAPI solution in 0.25% Triton/PBS (pH 7.2) at room temperature for 10 min, and subsequently washed 3 times in 0.25% Triton/PBS (pH 7.2) at room temperature for 5 min each. The samples were mounted serially in 30%, 50%, and 70% at room temperature for 10 min each and finally stored at 4℃ overnight. Signal detection was performed using a confocal laser scanning biological microscope (Olympus, Tokyo, Japan) according to the manufacturer's protocols.

Results

BrdU labels were observed in the gastric glands and vitellarium of the rotifers, but high intensities were detected in the vitellarium (Fig. 3-3). The BrdU uptake and the vitellarium development of individual rotifers within the CR and WF groups were almost similar.

The BrdU labels in the WF group were intensely detected in the vitellarium. In the WF group, the gastric glands and vitellarium volume as well as the BrdU labels gradually increased from the first 3-hour-feeding period to the following post-hatching stages. During the vitellarium development, the peripheral areas of vitellarium were heavily labeled with BrdU. The vitellarium development became distinct during 15 hph to 39 hph stages, and the peripheral labeling pattern of BrdU in the vitellarium was also clearly seen in these phases. With the increased vitellarium volume and BrdU labels, oocyte appeared in the WF group at 30 hph stage, when the vitellarium nearly occupied the entire post pseudocoelomic cavity. However, BrdU labels could not be detected in the fully developed oocyte appearing at 30 hph stage, but faint BrdU labels were observed in the periphery of the developing oocyte which became visible at 33 hph stage and still lacked a complete outer membrane. The number of eggs produced by the WF group at 33, 36 and 39 hph stages increased every 3 hours by one each. Although DAPI could label the eggs, BrdU labels were not observed in the eggs. In these females, an increase in egg number produced and the concomitant decrease in the vitellarium volume were observed, thus BrdU uptake would be also decreased during these periods. The volume of gastric glands and BrdU labels inside the glands were not clearly different among these females.

The CR group showed remarkably decreased BrdU labels compared to their WF counterparts. In the CR group, the gastric glands and vitellarium volume as well as BrdU labels from 6 to 27 hph stages were not conspicuous. The vitellarium volume of the CR group during that period remained unchanged, and was considerably smaller than those of the WF counterparts. However, the vitellarium volume as well as BrdU labels became distinct from 30 to 39 hph stages. These stages were comparable to the

younger WF group of 15 to 24 hph stages. The rates of vitellarium development and BrdU uptake in the adults of 30 to 39 hph stages were noticeably higher than those in the neonates of 6 to 15 hph stages during the 3-hour-feeding periods and the following CR periods.

Summary

BrdU was labeled in both the gastric glands and vitellarium of the rotifers, but dense labels were found in the vitellarium. In the WF group, the gastric glands, the vitellarium volumes, and the BrdU labels increased from the first 3-hour-feeding period to the following post-hatching stages. The BrdU labels were detected in the peripheral areas of the vitellarium. The oocyte appeared in 30-hph stage, but the BrdU labels were not detected in the developed oocyte. However, faint labels were observed in the developing oocyte, which appeared at the following 33 hph stage and still lacked a complete outer membrane. In the reproductive females of 33 to 39 hph stages, the number of eggs increased every 3 hours by one each, and the increase was accompanied by the reduced vitellarium volume as well as BrdU labels in the reproductive females.

In the CR group, the gastric glands and vitellarium volume were not conspicuous in the rotifers of 6 to 27 hph stages. However, the vitellarium volumes as well as the BrdU labels in CR females became distinct during 30 to 39 hph stages, the vitellarium volumes in those CR females were comparable to those in the younger WF females of 15 to 24 hph stages. The rates of vitellarium development as well as BrdU uptake in the adult rotifers were considerably higher than those in neonates during and after 3-hour-feeding periods in the CR group.

Section 2: Expression profiles of selected genes in the CR and WF groups of the same ages

Materials and methods

Animal culture and sampling

Culture conditions and sampling periods were the same as described in the previous Section 1. Thirty individuals were randomly sampled from each experimental culture of the CR and WF groups.

Total RNA extraction and cDNA synthesis

Total RNA extraction and cDNA synthesis were carried out as the same procedures described in Chapter1. The stock of first strand cDNAs were diluted 100 times and the diluted first strand cDNAs were used in qRT-PCR analyses.

Quantitative real-time RT-PCR (qRT-PCR) and Data analysis

Quantitative real-time RT-PCRs and data analyses were performed as the same procedures described in Section 1 of Chapter 2. The number of samples examined was three for each analysis.

Results

Among the genes related to DNA replication, the mRNA levels of *Msh6p* in eggs were not different from the level at 3 hph stage, but the level clearly decreased

during the first 3-hour-feeding period at 6 hph stages (Fig. 3-4A). After these periods, *Msh6p* was significantly up-regulated in the CR group during 9 to 30 hph stages. The mRNA level of *Pole* in eggs was lower than the level at 3 hph stage, but similar to the level at 6 hph neonates (Fig. 3-4B). Afterward *Pole* was significantly up-regulated in the CR group during 9 to 33 hph stages. *CDT1* mRNA level in eggs was higher than the levels at other stages (Fig. 3-4C). *CDT1* level in the CR group was up-regulated at 15 hph stage, but not significant ($p<0.07$). The mRNA level of *CDT1* in the CR group remained unchanged during all post-hatching stages. *CDT1* mRNA levels during 9 to 18 hph stages were not different between the CR and WF groups. After these periods, *CDT1* was significantly up-regulated in the WF group during 21 to 33 hph stages. *Pols* showed a similar expression pattern to *CDT1*, but *Pols* had significant up-regulation at 9 and 21 hph stages in the CR group (Fig. 3-4D). Afterward *Pols* mRNA levels were significantly up-regulated in the WF group from 24 to 33 hph stages, whereas the levels in the CR group remained unchanged.

In the group of genes related to cellular structure, transport, and division, the mRNA level of *BCCIPβ* in eggs was higher than the level at 3 hph stage, and the levels from 3 to 12 hph stages were not different (Fig. 3-4E). The significant up-regulation of *BCCIPβ* in the CR group was observed from 15 to 30 hph stages. *Lis1* mRNA level in eggs was higher, but the level subsequently decreased at 3 and 6hph stages (Fig. 3-4F). *Lis1* levels in the WF group were similar from 9 to 33 hph stages. *Lis1* in the CR group was significantly up-regulated from 9 to 21 hph stages. Afterward *Lis1* was down-regulated in the CR group from 24 to 33 hph stages. The mRNA level of *Dnahc3* in eggs was higher, but the level continuously decreased at 3 and 6 hph stages (Fig. 3-4G). After that, *Dnahc3* was significantly up-regulated in the CR group from 9 to 33

hph stages. *CaM64B* mRNA level was high in eggs (Fig. 3-4H). The level became increased at 3 hph stage, and the level subsequently decreased upon first feeding at 6 hph stage. *CaM64B* in the CR group was significantly up-regulated from 9 to 27 hph stages. The mRNA level of *Spnb1* in eggs was high, but the level continuously decreased at 3 and 6 hph stages (Fig. 3-4I). *Spnb1* in the CR group was significantly up-regulated from 9 to 24 hph stages and from 30 to 33 hph stages.

In metabolism group, the mRNA levels of *stom* between eggs and rotifers at 3 hph stage were not different, but the level became lower at 6 hph stage (Fig. 3-4J). Afterward *stom* was significantly up-regulated in the CR group from 9 to 27 hph stages. *SDHD* mRNA levels, like in *stom*, were not different between eggs and rotifers at 3 hph stage, but the level went down at 6 hph stage (Fig. 3-4K). *SDHD* mRNA level thereafter was significantly up-regulated in the CR group from 9 to 27 hph stages. The expression pattern of *Irp* in eggs and rotifers at 3 and 6 hph stages was similar to those of *stom* and *SDHD* (Fig. 3-4L). Afterward *Irp* was significantly up-regulated in the CR group from 9 to 24 hph stages. Its up-regulation in the WF group was also observed at 33 hph stage. *Amy2* mRNA level in eggs was low (Fig. 3-4M). After hatching the level became higher at 3 hph stage and then decreased upon feeding at 6 hph stage. Thereafter *Amy2* was significantly up-regulated in the CR group from 9 to 21 hph stage and at 27 hph stage.

Among the genes of other functions, the mRNA level of *TFPI* became higher at 3 hph stage, and the level decreased upon first feeding at 6 hph stage (Fig. 3-4N). *TFPI* thereafter was significantly up-regulated in the CR group from 9 to 27 hph stage. *Tsase* expression pattern from eggs to 6 hph stage was similar to *TFPI* expression (Fig. 3-4O). After these periods, *Tsase* was significantly up-regulated in the CR group from 9 to 27 hph stages. The mRNA level of *16S rRNA* was high in eggs, but the level successively

decreased at 3 and 6 hph stages (Fig. 3-4P). After that, *16S rRNA* was significantly up-regulated in the CR group from 9 to 27 hph stages. The expression of *14-3-3* from eggs to 6 hph stage was similar to that of *16S rRNA* (Fig. 3-4Q). The mRNA level of *14-3-3* was up-regulated in the CR group from 9 to 21 hph stages. Afterward, *14-3-3* mRNA level decreased in the CR group and the level remained unchanged, whereas the level in the WF group slightly turned up from 27 to 33 hph stages. Thus, the different mRNA levels were observed between the CR and WF groups.

Summary

Among 17 selected genes, 15 genes were significantly up-regulated in the CR group after imposing CR at 9 hph stage (Table 3-1). *CDT1* and *BCCIPβ* were exceptions. Of these 15 genes, 12 genes showed their significant up-regulated mRNA levels only in the CR group, whereas four genes showed in both CR and WF groups. The significant up-regulation of *BCCIPβ* occurred only in the CR group, but its significant up-regulation was started from 15 to 30 hph stages. *CDT1* showed its significant up-regulated mRNA levels only in the WF group from 21 to 33 hph stages. Three genes, *Pols*, *Lis1, Irp* and *14-3-3*, which had their significant up-regulation in both CR and WF groups, showed their up-regulated mRNA levels around 30 hph stage, when the oocyte appeared in the rotifers of the WF group.

Section 3: Discussion

In this Chapter, DNA synthesis in the CR and WF groups of the same ages was

studied by BrdU labeling method. Although BrdU labels were observed in the gastric glands and vitellarium of the rotifer, the vitellarium was found to be heavily labeled with BrdU, indicating that DNA synthesis mainly takes place in vitellarium of rotifers. In this experiment, the BrdU uptake and vitellarium development of individual rotifers within the CR and WF groups were nearly similar, supporting that the rotifers in both CR and WF groups were almost the same age and thus there have the same metabolic performance among individuals.

In the WF group from the first 3-hour-feeding period at 6 hph stage to the following stages, the gastric glands and vitellarium volumes, as well as the BrdU labels, gradually increased over time. This means that DNA synthesis continuously takes place in these organs of the WF groups. After the first feeding period at 6 hph stage, the vitellarium volume became distinct from 15 hph stage. The oocyte appeared at 30 hph stage and thus this stage could be regarded as an initial age of reproduction. Here, it is reasonable to conclude that the rotifers under the present experimental condition need about 27 h from the first feeding period to reach their age of reproduction. The BrdU labels were not detectable in the oocyte and it might be due to two reasons. First, the development of the oocyte had already completed before BrdU treatment and thus BrdU could not diffuse through the oocyte membrane to incorporate into the replicating DNA inside the oocyte. Second, DNA synthesis was absent in the oocyte. The latter is the most possible case, as the previous study reported that the oocyte is relatively inert during the entire growth period (Bentfeld, 1971b). It became evident when the developing oocyte appeared at 33 hph stage and the BrdU labels were nearly absent in the region of the developing oocyte. After producing the first egg at 33 hph stage, the number of egg increased every three hours by one each. Probably there might be an

interval of 3 hours between the previous and next egg production of the reproductive rotifers. In these females of ages from 33 to 39 hph stages, the increase in egg number and the concomitant decrease in the vitellarium volume were noticeable. There have been another similar reports on the reduction of vitellalrium volume in the rotifer *Asplanchna*, in which the nuclear number in the vitellarium decreased in the post-reproductive females and the oocytes developed individually at the expense of the vitellarium (Bentfeld, 1971b; Verdone-Smith and Enesco, 1982). The volumes of gastric glands as well as the BrdU labels were not different among these reproductive females.

In contrast to the WF group, the BrdU labels in both the gastric glands and the vitellarium were considerably lower in the CR group, indicating that DNA syntheses in these organs were inhibited in the CR group. As DNA synthesis is a prerequisite for nucleus division, these results are consistent with the previous study in which dietary restriction retards the nuclear division in the gastric glands and vitellarium of the rotifer *A. brightwell* (Verdone-Smith and Enesco, 1982). In the CR group, from the first 3-hour-feeding period at 6 to 27 hph stages the vitellarium volumes as well as the BrdU labels were not conspicuous. The vitellarium volumes in the CR group were uncomparable to and considerably smaller than those in their WF counterparts. If extra copying of vitellarium genome by DNA synthesis is necessary for reproduction in rotifers, here it is reasonable to assume that CR suppresses the reproduction of rotifers via the retardation of DNA synthesis in the vitellarium. However, from the second 3-hour-feeding period at 30 to 39 hph stages the rate of vitellarium development as well as of BrdU labeling became higher in the CR group. The vitellarium volumes and BrdU labels in these CR females at 30 to 39 hph stages were comparable to those in their younger WF females at 15 to 24 hph stages, indicating that CR represses reproduction

in the reproductively active rotifers, but these CR rotifers maintain the same capability of reproducing as occurred in their younger WF ones. This also supports the hypothesis that CR animals, but not all, increase life span at the expense of reproduction and this life span extension may be due to the retardation of reproductive senescence in the CR animals. Regarding the rapid increase in vitellarium volume as well as in the BrdU labels of the adult CR females after the second 3-hour-feeding period, it is conceivable that CR can not prevent the reproduction of rotifers completely instead it slows down the rate of reproduction. Even under CR, the rate of the vitellarium development was remarkably faster in the adults than in the neonates, showing the reproductive readiness in the adult rotifers regardless of an adverse environmental condition.

The transcripts of the selected genes seemed to be maternally transmitted via extra copying of vitellarium genome except *Amy2*. It has been suggested that not only ribosomes, but also mRNA is supplied to the oocyte of the rotifer *A. brightwelli* by the vitellarium (Bentfeld, 1971b). Although the transcript levels showed up and down just after hatching at 3 hph stage where the rotifers were not fed, the levels after this stage in the WF group were relatively lower than the levels in eggs. Overall, all transcript levels became lower during the first 3-hour-feeding period at 6 hph stage. Except *CDT1*, *Pols* and *BCCIPβ*, all the selected genes in the CR group generally had a higher significant up-regulation from 9 to 21 hph stages. Thereafter, the transcript levels were sharply decreased from 24 to 33 hph stages, but most genes still maintained their up-regulation in the CR group. That decrease in the transcript levels may be due to the translational regulation of the transcripts and this regulation might be important when the rotifers enter the age of reproduction. Thus, the expression levels of the selected genes from 24 to 33 hph stages might be critical for regulating the metabolic processes of the CR

rotifers, because reproduction appeared during this period. As CR suppresses the reproduction in the rotifer *B. plicatilis*, the changes in the expression level of selected genes during the reproductive period are attributable to the longer life span of the rotifer at the expense of reproduction. If this is true, then up-regulation of *CDT1*, *Pols* and *14-3-3* in the adult WF rotifers and down-regulation of *Lis1* in the adult CR rotifers might contribute to the differences in reproductive performance of the rotifers in response to food variability. Based on the present study, the reproduction in the adult WF rotifers might be due to the increase up-regulation of *CDT1* and *Pols*. Their expression in the adult CR rotifers might be suppressed during DNA repairing processes, because DNA repair is prerequisite for redefining the maternal genome that is going to be transferred to the offspring. In this study, *Msh6p* and *Pole* most likely to perform DNA repair and their significant up-regulation in the adult CR rotifers might reflect DNA repair exists and during these periods DNA synthesis activated by *CDT1* and *Pols* is inhibited. On the other hand, *BCCIPβ* up-regulation might be the most possible factor that negatively controls the *CDT1* and *Pols* activities in DNA synthesis. If DNA synthesis in the vitellarium itself is the first step of reproduction in the rotifers, *Msh6p*, *Pole*, *CDT1*, *Pols* and *BCCIPβ* are responsible during this step and they are the first line regulators of reproduction in the rotifers. CDT1 seemed to be most responsible for reproduction, because it is fundamentally important for DNA synthesis in vitellarium. *Lis1* down-regulation in the adult CR rotifers is attributable to the delay of oocyte development. Therefore, Lis and its partners function together in oocyte maturation might act as the second line regulators of reproduction in the rotifers.

CHAPTER 4

General discussion and conclusion

As a first attempt to study molecular mechanisms involved under CR, CR-induced differentially expressed genes were investigated in *B. plicatilis* by SSH technique. Among the differentially expressed genes obtained by SSH, DNA synthesis- and cell cycle-related genes are quite interesting because rotifers are eutelic. Therefore, 17 genes including DNA synthesis- and cell cycle-related genes, were further selected to be studied their detail expression patterns by qRT-PCR analyses. As a CR-like condition would be occurred in the stationary phase of population growth, the expression levels of the selected genes were also studied in CR-like stationary phase. As expected, 14 genes were consistently and significantly up-regulated in both CR and CR-like stationary phase, indicating their expressions were affected by a limited food condition. Of other 3 genes, *CDT1*, *Pols* and *Spnb1*, even though *CDT1* and *Pols* were up-regulated under CR, their expressions were down-regulated in the CR-like stationary phase. *Spnb1* showed ambiguous expression patterns in the CR experiment; *Spnb1* had significant up-regulation in both CR and WF groups. *Spnb1* expression seemed not to be CR-specific, as its expression level was also higher in the exponential phase of population growth. Although it should be similar, the contradictory expression pattern of two DNA synthesis-related genes, *CDT1* and *Pols*, between CR and CR-like stationary phase still remained as an interesting question. However, qRT-PCR further approved the up-regulation of other 14 selected genes under a limited food condition. Their expression could be represented as population-wide expression, as the studies were conducted in the rotifer populations of various ages.

Subsequent qRT-PCR analyses conducted in the rotifer populations of the same ages gave more clear and decisive expression patterns of the selected genes. At the same time, study on DNA synthesis by BrdU labeling revealed a potential role of vitellarium in the rotifers in response to CR. As the vitellarium is a reproductive organ, a clear increase in the BrdU labels in the vitellarium as well as the vitellarium volume of the WF rotifers indicates that a well-feeding condition stimulates the rotifers to reproduce via a rapid DNA synthesis in the vitellarium. The delay in the vitellarium development under CR is attributable to the suppression of DNA synthesis in the vitellarium. Because DNA synthesis is prerequisite for reproduction, it is possible that the CR rotifers suppress the reproduction via the direct inhibition of DNA synthesis in the vitellarium. BrdU labeling made clear that the CR rotifers suppress their reproduction through the inhibition of vitellarium DNA synthesis.

Based on the putative functions of the selected genes, *Msh6p*, *Pole*, *CDT1* and *Pols* might directly regulate DNA synthesis in the vitellarium. Msh6p and Pole work together for DNA repair, especially mismatch repair that might occur before or during DNA synthesis. During repairing period other DNA synthesis processes are likely to be inhibited under DNA repair mechanisms. Thus, in the present study, CDT1 and Pols in the CR rotifers are most probably inhibited during DNA repair. If CDT1 is most responsible for polyploidization in the vitellarium, the most possible inhibitor of CDT1 is BCCIPβ because its interacting protein BRCA2 inhibits polyploidization (Sagulenko et al., 2007). Here, it is reasonable to conclude that the expression patterns of *Msh6p*, *Pole*, *CDT1*, *Pols* and *BCCIPβ* affect the reproductive performances in the CR and WF rotifers. Adult-specific up-regulation of *CDT1* seems to be the most responsible factor for reproduction of rotifers and its down-regulation in the CR rotifers might be directly

inhibited by *BCCIPβ* up-regulation in the present study. Therefore, these five genes could be regarded as the first line regulators and they would regulate the rotifer reproduction through vitellarium DNA synthesis.

Lis1, Dnahc3, Spnb1 might regulate the reproduction of rotifers during oocyte determination, development and maturation. Therefore, these three genes might act as the second line regulators and they would regulate the rotifer reproduction during oogenesis. *Lis1* down-regulation in the adult CR rotifers is the most possible factor that hampers the oogenesis until the vitellarium DNA synthesis is completed. Swan *et al.* (1999) proposed a model in which *Drosophila* Lis1 (DLis1) was emphasized as a key regulator for nurse-cell-to-oocyte transport during oocyte growth. Because the rotifer vitellarium is analogous to the nurse cell of *Drosophila*, the similar mechanisms of *Dorsophila* oogenesis might be occurred during the rotifer oogenesis. If this is true, down-regulation of *Lis1* in the adult CR rotifers prevents oogenesis and this delay would last until the extra copying of vitellarium genome is completed enough for oocyte development. According to the resource-allocation theory, these genes are key regulators that regulate the energetic trade-off between reproduction and somatic maintenance of the rotifers under CR.

Other genes observed in the present study are also important and their expression might contribute to the fitness of the CR rotifers. For example, *16S rRNA* expression is down-regulated under hypoxia and oxidative stress (Crawford *et al*., 1997; Kowaltowski and Vercesi, 1999; O'Hara *et al.*, 2003). Thus, *16S rRNA* up-regulation in the CR rotifers means the CR rotifers are not under hypoxia and oxidative stress. Whether 16S rRNA directly participates in regulating cellular oxygen level is unknown. However, *SDHD* up-regulation might confer a normal cellular oxygen level to the CR

rotifers, because SDHD has been proposed to function in cellular hypoxia (van Nederveen *et al.*, 2003). 14-3-3 has several isoforms and thus it is hard to know the exact roles of its up-regulation in the CR rotifers. There have been other reports in which 14-3-3 protein (also knows as YWHA) participates in oocyte maturation in mice (Snow *et al,* 2008) and amphibians (Margolis *et al.*, 2003; reviewed in Darling *et al.*, 2005). Thus, 14-3-3 down-regulation in the adult CR rotifers is attributable to the delay in oocyte development. The beneficial effects of *Tsase* expression on the body maintenance of organisms have been discussed in the chapter sections. Besides, Tsase also has possible roles in reproduction of organisms. In *C. elegans*, mutations in *msh-2* (another DNA mismatch repair gene) cause a reduced fertility and msh-2 gene has been know to be disrupted by a transposable element (TE), which encodes Tsase (Degtyareva et al., 2002), supporting the possible effects of *Tsase* up-regulation on the reproductive suppression in the CR rotifers.

The primary role of CR seems to postpone reproductive senescence upon the somatic maintenance, thereby animals gain an increased chance of survival with a reduced intrinsic rate of senescence. Based on the previous studies and the present findings, it is conceivable here that these aspects of CR may also be found in the calorie-restricted rotifer of *B. plicatilis* (Ishikawa strain). Although the mechanisms by which CR exerts its effects on life span extension in various organisms are still poorly defined and largely debatable, the expression patterns of the differentially expressed genes and the reproductive performance of the CR rotifers in this study shed light on a possible molecular mechanism by which the rotifers extend their life span at the expense of reproduction during CR. Under this molecular mechanism, reproduction of *B. plicatilis* during CR is most likely to be orderly controlled at two developmental

processes: first, at vitellarium DNA synthesis and second, at oogenesis. Therefore, the expression levels of the genes participate in these processes are the key factors for the life span extension at the expense of reproduction in *B. plicatilis*. Other differentially expressed genes in the present study are also indispensable for the rotifer in maintaining their metabolic processes under CR. It is likely that there are other genes remaining out of the present study due to differences in experimental procedures. However, the present study on CR-induced gene expression in *B. plicatilis* gives not only information about the differentially expressed genes and their expression patterns in response to CR, but also it sheds light on a possible molecular mechanism which regulates the reproduction of *B. plicatilis* during CR. If the mechanisms by which CR confers robust physiology to organisms are evolutionarily conserved, the present study would be part of support for understanding these mechanisms.

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Table 1-1. Differentially expressed genes in calorie-restricted *Brachionus plicatilis* as revealed by suppression subtractive hybridization (SSH)

Putative gene	Abbreviation	Accession [a] number	Query size (bp)	Accession [b] number	E value	Percent identity
Genes related to cellular structure, transport and division						
Lissencephaly-1	↑ *Lis1*	AB491778	288	XP_392399	7e-18	69
Dynein heavy chain domain 3	↑ *Dnahc3*	AB491771	481	XP_001255916	6e-43	59
Beta 2 tubulin	↑ *tub*	AB491792	301	AAX09675	2e-37	97
Microtubule-associated protein EB 1	↑ *MT-EB1*	AB491780	230	BAC05521	6e-08	61
BRCA2 and CDKN1A-interacting protein, isoform BCCIPβ	↑ *BCCIPβ*	AB491767	676	XP_001863388	2e-17	31
Calmodulin (synthetic construct)	↑ *CaM64B*	AB491768	427	AAD34268	1e-11	40
Spectrin beta chain	↓ *Spnb1*	AB491799	138	XP_879610	0.090	53
Elongation factor Tu mitochondrial	↓ *Ef-tu*	AB491795	319	EAT39702	1e-32	62
Proteasome regulatory particle, atpase-like protein	↓ *rpt*	AB491798	228	NP_001022114	2e-33	93
Genes related to DNA replication						
CDT1 protein	↑ *CDT1*	AB491769	303	XP_969028	0.001	40
Mismatch repair protein in mitosis and meiosis	↑ *Msh6p*	AB491779	424	NP_010382	8.6	38
DNA polymerase epsilon	↑ *Pole*	AB491784	495	EAT45963	7.3	41
DNA polymerase sigma	↑ *Pols*	AB491785	239	NP_001012968	2e-26	73

E value expectation value, the number of the different alignments with scores equivalent to or better than raw score (S) that are expected to occur in the database search by chance. [a] registration number, [b] database number.

Up- and down-arrows represent the up- and down-regulation of their corresponding genes confirmed by semi-quantitative reverse transcription-PCR (RT-PCR). * Gene showed no mRNA level changes in the CR and WF groups during both RT-PCR analyses.

Table 1-1. (*Continued*)

Putative gene	Abbreviation	Accession [a] number	Query size (bp)	Accession [b] number	E value	Percent identity
Genes related to metabolism						
Stom protein	↑ *stom*	AB491788	564	AAH91908	3e-43	54
Galactose-4-epimerase, UDP	↑ *Gale*	AB491773	432	XP_001606062	2e-22	66
Glycogen phosphorylase	↑ *Glase*	AB491775	153	NP_001001904	2e-14	76
β-galactosidase	↑ *Glb*	AB491774	262	XP_792349	3e-11	49
2-isopropylmalate synthase	↑ *IPS2*	AB491776	343	YP_609867	0.76	78
Succinate dehydrogenase complex subunit D	↑ *SDHD*	AB491786	180	AAW70035	8.4	50
Iron regulatory protein	↑ *Irp*	AB491777	412	AAR15297	3e-09	51
Peptidylglycine-hydroxylating monooxygenase	↑ *Phm*	AB491782	312	NP_477225	1e-12	47
Amylase 2, pancreatic	↓ *Amy2*	AB491794	441	XP_575020	2e-50	59
Alpha amylase	↓ *αAmy*	—	296	P91778	4e-17	70
Phosphatidylserine/phosphatidylglycophosphate /cardiolipin synthase	↓ *PS/PGp/Cl*	AB491797	251	ZP_01368877	8.4	25
Oligosaccharyl transferase STT3 subunit homolog	↓ *stt3*	AB491800	179	XP_001061492	1e-23	86
Mitochondrial processing peptidase beta subunit	↓ *mtPP*	AB491796	332	EAT43097	3e-39	64
Cytochrome *c* oxidase subunit II	∗ *COX II*	—	473	NP_008174	4e-37	55

Table 1-1. (*Continued*)

Putative gene	Abbreviation	Accession [a] number	Query size (bp)	Accession [b] number	E value	Percent identity
Genes related to other functions						
Tissue factor pathway inhibitor	*TFPI*	AB491790	372	XP_585593	6e-14	39
Serine protease	*Ser*	AB491787	521	EAT46744	5e-08	38
Multifunctional 14-3-3 family chaperone	*14-3-3*	AB491766	247	ABX80390	2e-34	85
Serine/threonine phosphatase	*STPP*	AB491789	174	AAD01260	3e-26	98
Serine/threonine protein kinase with TRP repeats	*PK-TRP*	AB491783	200	YP_593054	8.6	38
Transposase	*Tsase*	AB491791	380	NP_602772	1.3	54
Mitochondrial partial 16S rRNA gene	*16S rRNA*	—	459	AP009407	6e-59	83
Genes related to transcription						
Zinc finger protein	*Znf*	AB491793	307	FAA00107	5e-05	48
EBF protein	*EBF*	AB491772	304	XP_688771	5.4	42
Genes related to RNA biosynthesis						
NOL1/NOP2/Sun domain family 2 protein	*NSUN2*	AB491781	234	XP_002190161	3e-14	53
Unknown						
Conserved hypothetical protein	*CHP*	AB491770	261	EEC00668	0.45	33

Table 1-2. Nucleotide sequences of primers used in semi-quantitative reverse transcription-PCR (RT-PCR)

Gene	Forward primer (5' to 3')	Reverse primer (5' to 3')
Lis	CTTTTTGCTGTCCGTCA	GCGCTTTATTGTAATGTAAGT
Dnahc3	TAAACGCGAACGATACAA	TTGTGTTGGCTTAGAAAGAA
tub	TCTGTGCTGGACGTGAT	ATGTCGTACAAAGCCTCATT
MT-EB	GCAGCTAACACCTCGAA	CACCTCAATGTCCCTCAA
BCCIPβ	TGAAGACCAGGTTGAAGAT	AGCTGAATCTTCACCAAGTT
CaM64B	CGCCGTCCAAATCA	CAATAGTTGGCAAAATTAGAGAT
CDT1	GAGAAAATTTTCATCGACTTC	TCACTTATTTCGGGCAA
Msh6p	CCCAACCACGCAA	AGTCTGGTCAGCTCGTT
Pole	ACTGGATACTAAGACCGTCAA	ATCCATTTGGCCGAT
Pols	GGTGATGGTGCCGA	GCTGCCGCCGTT
stom	TCAGAAGGAAATCAAAAT	ATTTTACGTCTTTCACTTCT
Gale	CGGCATCGGCTACT	AGCCTCATGGAAAAATATCTA
Glase	CGCGTGTGGTCAT	ACTCTGTAGTTCTCCAAGA
Glb	ACTACACTTGCTGATCCAA	ATTATCTAAAGTGACATTTGACA
IPS2	GTAGAACGTCAGCTTCCCAA	AGGGGTCGCGCAA
SDHD	AGTCCGTAACTGCAGTTT	AACTCGAGTTCCAGTGAA
Irp	ACTGAAAAGTTTAACATCCTTA	GGATCAATTTTAAGAAATATAATAA
Phm	TGGTTTGATTGCGCTAT	AACCGGACCTAGTTTGTAGTT
TFPI	CGAAATTTAAATAAAATAAA	CATATTCCAGGTTTCTT
Ser	ATGTGAATCAAATGTGACAGAT	CATAGATTCTTTGGTCGGAA
14-3-3	ATCGGCGAAGACAGAA	GTTTGGCCAAGTGACAA

Table 1-2. (*Continued*)

Gene	Forward primer (5' to 3')	Reverse primer (5' to 3')
STPP	CCGGGACCGCAT	AATATTTGTGAGTCGACCAGT
PK-TRP	GAGAGCGGCGACTGTCA	ACTTGGCCTGTCGCTTGTT
Tsase	CAAGAATGTGCATAGTTTTAC	AATTTTGAAAATTTATTTGTTAG
16S rRNA	CTAACACATCTAAATATCGAATA	CAGTGGGCAGAAGATA
Znf	AGCAGCAGATCAATCAACTA	TCGGAACTGCTGCAT
EBF	CCTAGTCATTGGCGAATCA	ACCTGGACCATATTGTTCATGTA
NSUN2	GAATAGCCAAACGAGCA	AGTGTCTAAAATTGCCTTGTT
CHP	CCAAATTTATATGATTCATGCAT	GACCAAACCAAACTCTTGAA
Ef-tu	GTGTGGAGAGAGGCGTA	ACAATTGAGGTTGGTAGGTT
Spnb1	AGGAAATGGTGGTCAGTAT	GTCTTGTTGGTAATATCGCTA
rpt	CTGTTGGCTAGAGCTGTT	TGAATTTCTCTATCAGCTGAA
Amy2	GCGAAATCATCACTCACA	CGCTAATTATGTCACAGTATTGA
αAmy	GCGAAATCATCACTCACAA	CGATTGACCATTGGCAA
PS/PGp/Cl	GAGTTTCATTTTGTTGGAT	CGAAGACCTGTTAACTGA
stt3	GAGAGAGCTGGACGTGGACTA	CCCTTGCGGCGTGTAA
mtPP	CTGGTAGTGAGATCCGAGT	GGGAAATCATAAAGTCATCTATT
COX II	AATCTCTCTACATTGACCA	CATATTTTAGAGACTATCTGAACT
EF-1α[*]	TGGCACATCACAAGCCGAT	GATACCGGCTTCGAATTCACC

* Internal standard

Table 1-3. Numerical data for RT-PCR analyses of differentially expressed genes retrieved from Electrophoresis Documentation and Analysis System

Gene	Increased level	
	1st (CR/WF)	**2nd (CR/WF)**
Lis1	4.20	28.32
Dnahc3	1.20	2.41
tub	1.25	1.72
MT-EB1	2.29	1.14
BCCIPβ	8.19	1.02
CaM64B	2.49	2.24
CDT1	4.10	1.40
Msh6P	2.91	2.19
Pole	1.66	2.36
Pols	1.21	1.07
stom	1.06	1.14
Gale	1.33	1.42
Glase	1.26	1.53
Glb	1.10	1.86
IPS2	3.01	1.03
SDHD	1.46	1.27
Irp	1.83	1.60
Phm	1.49	1.52
TFPI	1.10	1.40
Ser	1.64	1.85
14-3-3	1.27	1.45
STPP	1.37	1.11
PK-TRP	2.28	1.08
Tsase	20.87	6.52
16S rRNA	1.64	1.04
Znf	2.29	1.33
EBF	2.01	1.04
NSUN2	2.47	7.54
CHP	1.47	1.73

Table 1-3. (*Continued*)

Gene	Decreased level	
	1st (CR/WF)	2nd (CR/WF)
Ef-tu	0.56	0.76
Spnb1	0.85	0.57
rpt	0.29	0.58
Amy2	0.57	0.93
αAmy	0.67	0.67
PS/PGp/Cl	0.62	0.70
Stt3	0.68	0.88
mtPP	0.87	0.89
COX II [a]	1.00	1.00
EF-1α *	1.00	1.00

* Internal standard

[a] Gene showed unchanged mRNA levels during the analyses.

Table 2-1. Nucleotide sequences of primers used for quantitative real-time RT-PCR (qRT-PCR) analyses

Gene	Forward primer (5' to 3')	Reverse primer (5' to 3')
Msh6p	GCCGCACCAACATCAACA	TGCTGGCAAACGTAAAGCTTT
Pole	GTCAACAATGTCGTCAATGCCATA	CGATGCCTTGAAGCTGGTTTC
CDT1	TTGTCAAAAAGTTGCATCAGGAATT	TGCTGTCCACGGGAACGT
Pols	CGCTCGGCGCCAATC	AGCGGCCGTAGAACTCGAA
BCCIPβ	TAAAATCCCACACAAACCAAGAAAA	CTTTCGTTGACAATATAGGAACATTTAGG
Lis1	GAGCTGAACCGGGCCATA	AGTCCAGCGTTTGACGGTAGTT
Dnahc3	AGGAGATTGAGTTTATGGAAATGATGT	TTTCTCCTGCCAAGCCTGAT
CaM64B	CTCCTCAGTCATCAGCTTCAAAAA	GCGACTTTCCACTGTCCAAAA
stom	GGACCTGGCCTATTTTTCATTGT	AGTGACTGCTCTTAAATCCACGTTT
SDHD	TCCCGGAATCAAAGCGAGTAG	GCCGGTCAACATTGGACTATTG
Irp	GAAGTAGAGACTGATTCTGGCATTAAATTC	CCGCCATTTTTAATATAATTAAGCTCAAC
TFPI	CGAACAAATGCGAGCAGTTTAC	GAACAATCCTCCATTGAATGAAAA
14-3-3	TACTGTGATTGACGACTCGCAA	TGGCTCTTGGCGATCTCG
Tsase	GTTTTACATGGATAAGTGAAAATGATGACT	GACTATTTTCATACACCACTCTCAAACAA
16S rRNA	AACCTGTCTTGCAACGGTTTAAA	CCTCGATGTTGGATTATCTTTTCATA
Spnb1	AACAACTGGATTAGCCGAACTCAA	CGCTAGGCGATCCGTTTGTA
Amy2	TGGCCTGGCCCTATGCTAAT	TCCGAGCCAATCTTGTGAAAGT
*EF-1α**	CAAGAACATGATCACTGGCACATC	CCCACACCAGCAGCTACGAT

* Internal standard. Refer to Table 1-1 for abbreviations of genes.

Table 3-1. Summary of the significance levels and expression patterns of the selected genes

Gene	Age of the rotifers (hph, hour-post-hatching)									
	6-hph*	9-hph	12-hph	15-hph	18-hph	21-hph	24-hph	27-hph	**30-hph*¶**	33-hph
(A) *Msh6p*	—	p<0.05	p<0.001	p<0.001	p<0.01	p<0.01	p<0.05	p<0.05	p<0.01	—
(B) *Pole*	—	p<0.001	p<0.01	p<0.001	p<0.001	p<0.001	p<0.01	p<0.01	p<0.001	p<0.001
(C) *CDT1*	—	—	—	—	—	p<0.01	p<0.01	p<0.001	p<0.001	p<0.001
(D) *Pols*	—	p<0.001	—	—	—	p<0.05	p<0.01	p<0.001	p<0.01	p<0.05
(E) *BCCIPβ*	—	—	—	p<0.01	p<0.01	p<0.01	p<0.05	p<0.01	p<0.01	—
(F) *Lis1*	—	p<0.05	p<0.01	p<0.05	p<0.01	p<0.001	p<0.001	p<0.05	p<0.01	p<0.01
(G) *Dnahc3*	—	p<0.05	p<0.001	p<0.01	p<0.01	p<0.001	p<0.01	p<0.01	p<0.01	p<0.001
(H) *CaM64B*	—	p<0.001	p<0.001	p<0.001	p<0.001	p<0.01	p<0.01	p<0.001	—	—
(I) *Spnb1*	—	p<0.01	p<0.05	p<0.001	p<0.01	p<0.001	p<0.05	—	p<0.05	p<0.01
(J) *stom*	—	p<0.001	p<0.001	p<0.001	p<0.001	p<0.01	p<0.001	p<0.05	—	—
(K) *SDHD*	—	p<0.01	p<0.05	p<0.001	p<0.05	p<0.001	p<0.01	p<0.01	—	—
(L) *Irp*	—	p<0.01	p<0.001	p<0.01	p<0.001	p<0.001	p<0.01	—	—	p<0.05
(M) *Amy2*	—	p<0.01	p<0.001	p<0.001	p<0.001	p<0.001	—	p<0.01	—	—
(N) *TFPI*	—	p<0.01	p<0.05	p<0.01	p<0.01	p<0.01	p<0.01	p<0.01	—	—
(O) *Tsase*	—	p<0.001	p<0.001	p<0.001	p<0.001	p<0.001	p<0.001	p<0.001	—	—
(P) *16S rRNA*	—	p<0.001	p<0.001	p<0.05	p<0.01	p<0.001	p<0.01	p<0.05	—	—
(Q) *14-3-3*	—	p<0.01	p<0.01	p<0.001	p<0.01	p<0.001	—	p<0.01	p<0.01	p<0.001

Yellow and green colors stand for the accumulated mRNAs in the CR and WF groups, respectively. *Feeding periods in the CR group. ¶ Oocyte appeared in the WF rotifers, — no different levels. Refer to Table 1-1 for abbreviations of genes.

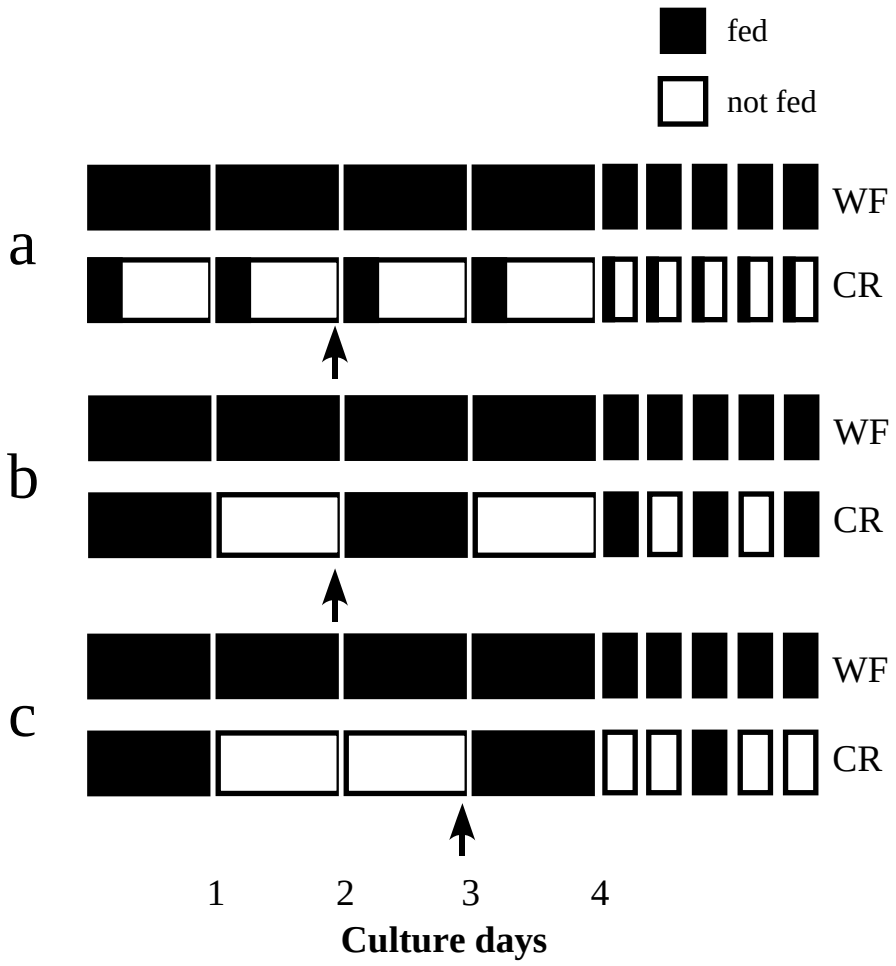


Fig. 1-1. Feeding regimens used in cDNA subtraction (a) and semi-quantitative reverse transcription-PCR (RT-PCR) analyses (b, c). cDNA subtraction was preformed between the calorie-restricted (CR) and well-fed (WF) groups. Subsequent validation of the differentially expressed genes was carried out two times by RT-PCR analyses. In panel (a), the CR group were fed 3 h/day. The CR groups in panels (b) and (c) were fed every two and three days, respectively. The WF groups were fed *ad libitum*. Sampling points are indicated by arrows.

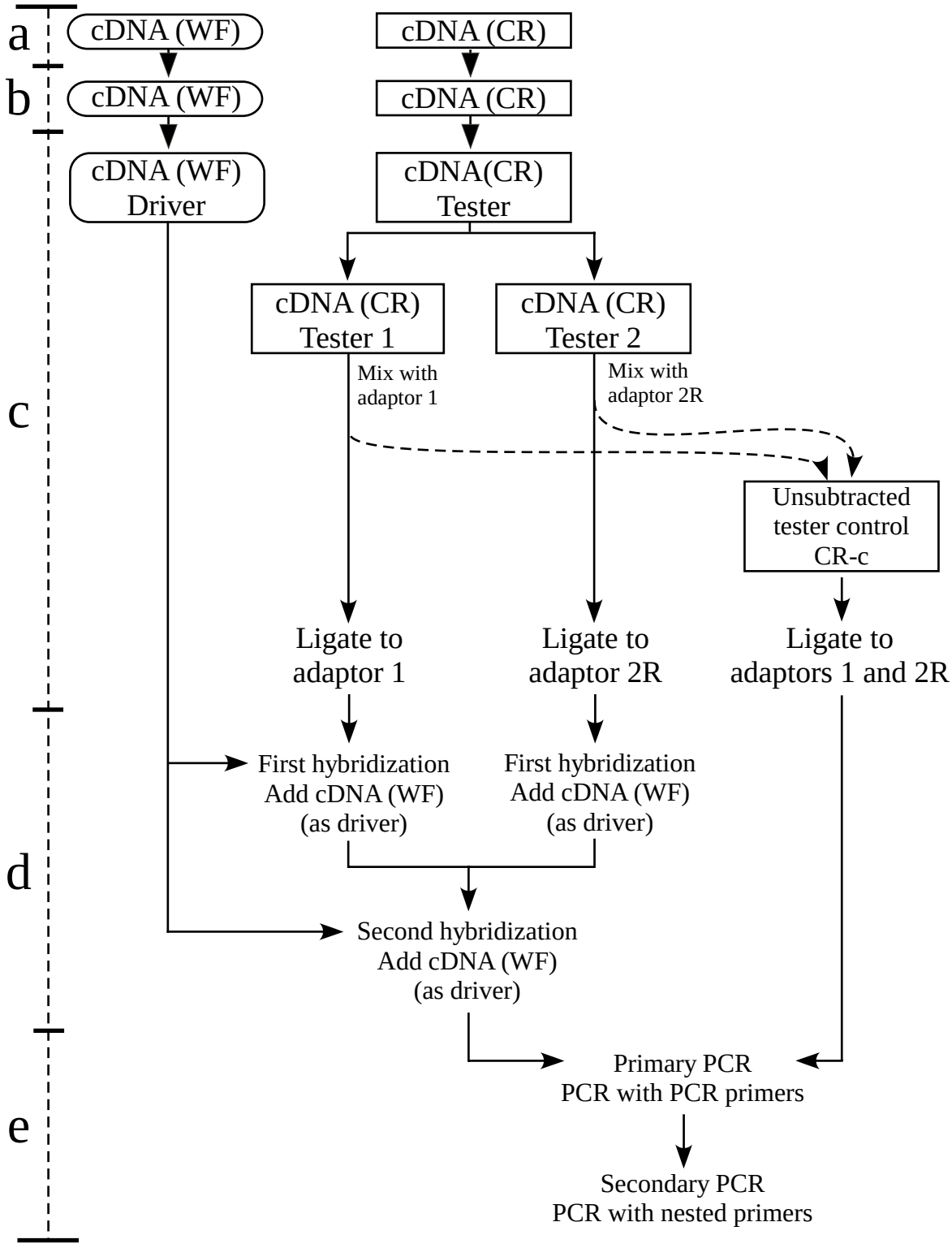


Fig. 1-2. Basic steps in suppression subtractive hybridization (SSH) technique (a-e) and type of cDNA subtraction applied to the present study. SSH technique basically consists of cDNA synthesis (a), enzyme digestion (b), adaptor ligation (c), hybridization (d), and PCR amplification (e). The present study was focused on calorie restriction (CR). cDNAs prepared from the CR samples were used as a tester, whereas those prepared from the control well-fed (WF) samples were used as a driver. The tester cDNAs were subtracted from the driver cDNAs. CR-c, CR unsubtracted tester control; PCR, polymeric chain reaction; cDNA, complementary DNA.

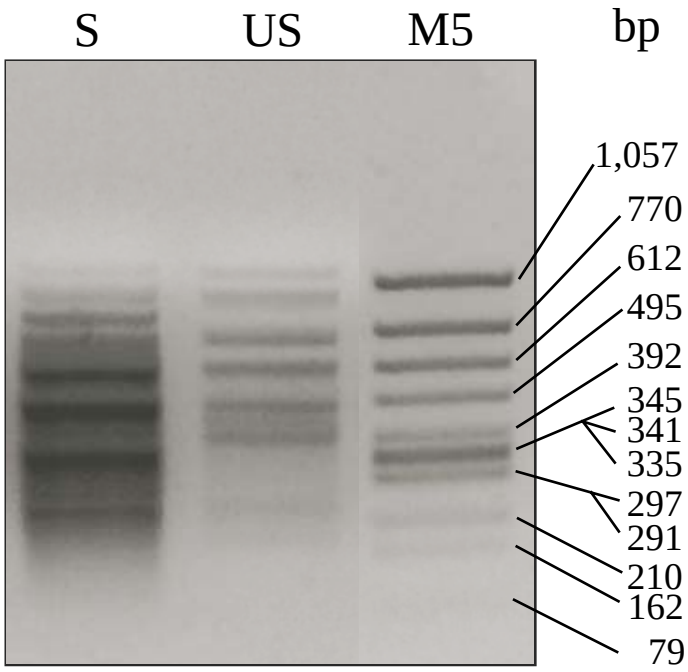


Fig. 1-3. Results of suppression subtractive hybridization (SSH). The cDNAs of the calorie-restricted samples were subtracted from those of the well-fed samples. Subtracted PCR products were run on a 2% agarose gel containing ethidium bromide. S, subtracted PCR products; US, unsubtracted PCR products; M5, molecular weight marker 5; bp, base pair.

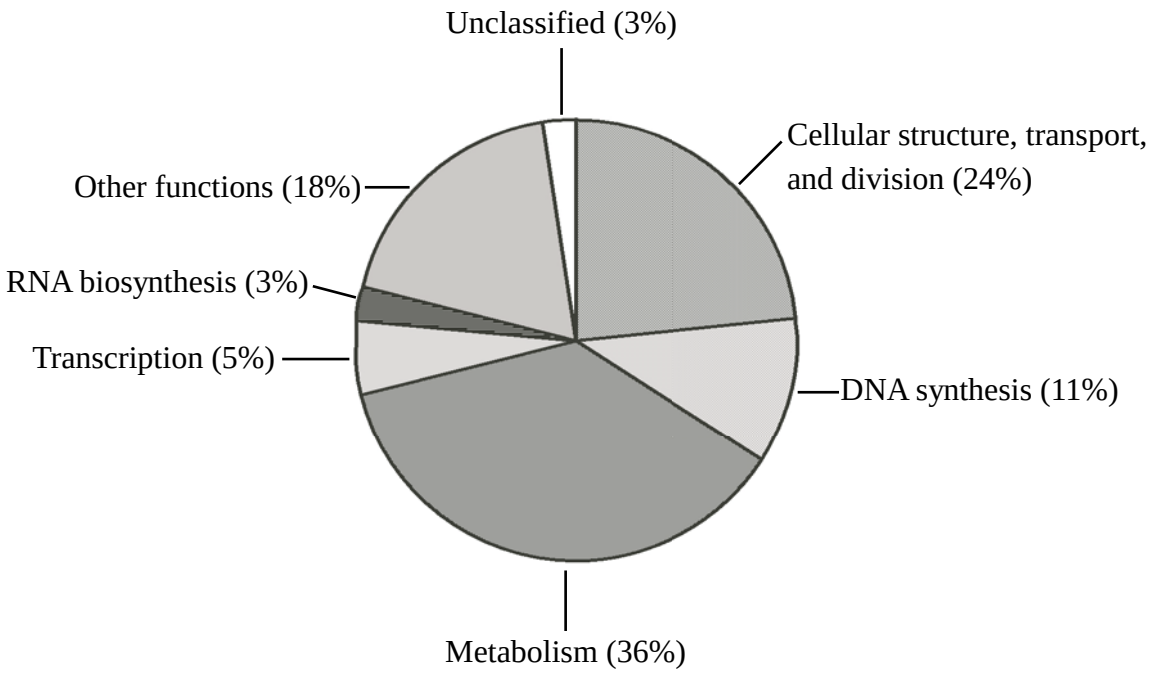


Fig. 1-4. Gene ontology analysis on 38 differentially expressed genes in calorie-restricted *Brachionus plicatilis* by functional classification for predicted proteins.

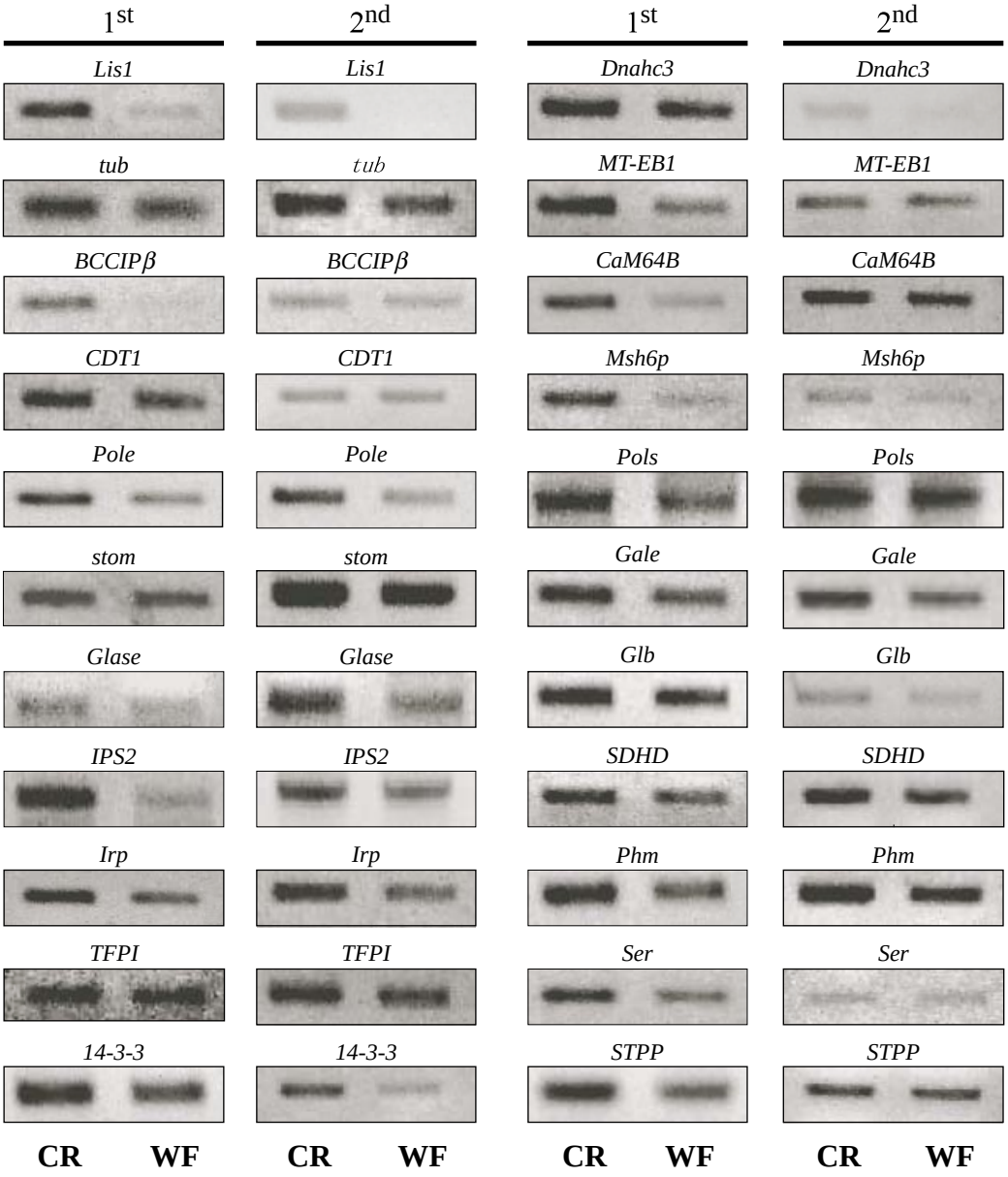


Fig. 1-5. Semi-quantitative reverse transcription-PCR (RT-PCR) analyses for verifying the expression of CR-induced differentially expressed genes revealed by suppression subtractive hybridization (SSH). RT-PCR was performed two times. In the 1st and 2nd experiments, the rotifers of the calorie-restricted (CR) groups were fed every 2 and 3 days, respectively, whereas those of the well-fed (WF) groups were fed *ad libitum*. For the gel electrophoresis, the equal amounts of the late exponential phase (one cycle ahead of the plateau phase) PCR products were run on a 2% agarose gel containing ethidium bromide. Genes marked with an asterisk were down-regulated in the CR groups during both experiments. The boxed gene, *COX II*, showed its unchangeable mRNA levels in the CR and WF groups during both experiments. Elongation factor-1α gene (*EF-1α*) was used as an internal standard for normalizing the mRNA levels of the target genes. Refer to Table 1-1 for abbreviations of genes.

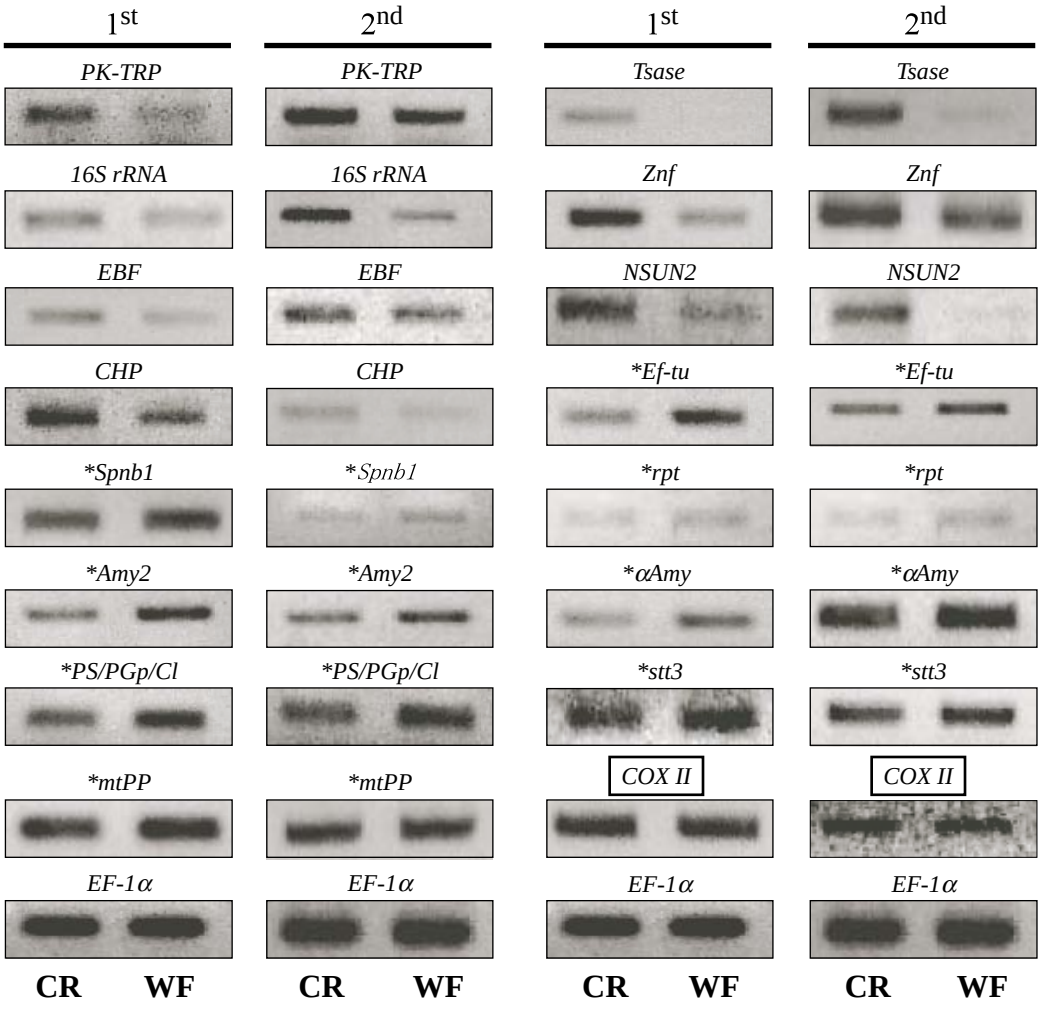


Fig. 1-5. *(Continued)*

Fig. 2-1. Feeding and sampling schedules for quantitative real-time RT-PCR (qRT-PCR). qRT-PCR was employed to determine the accumulated mRNA levels of selected genes in 8 consecutive culture days. The mRNA levels were analyzed and compared between the calorie-restricted (CR) and well-fed (WF) groups with individuals of various ages. The rotifers in the CR group were fed 3 h/day, whereas their counterparts in the WF control group were fed *ad libitum*. Three experimental replications were performed for both groups and 30 individuals were randomly sampled from each replication. Sampling points are indicated by arrows.

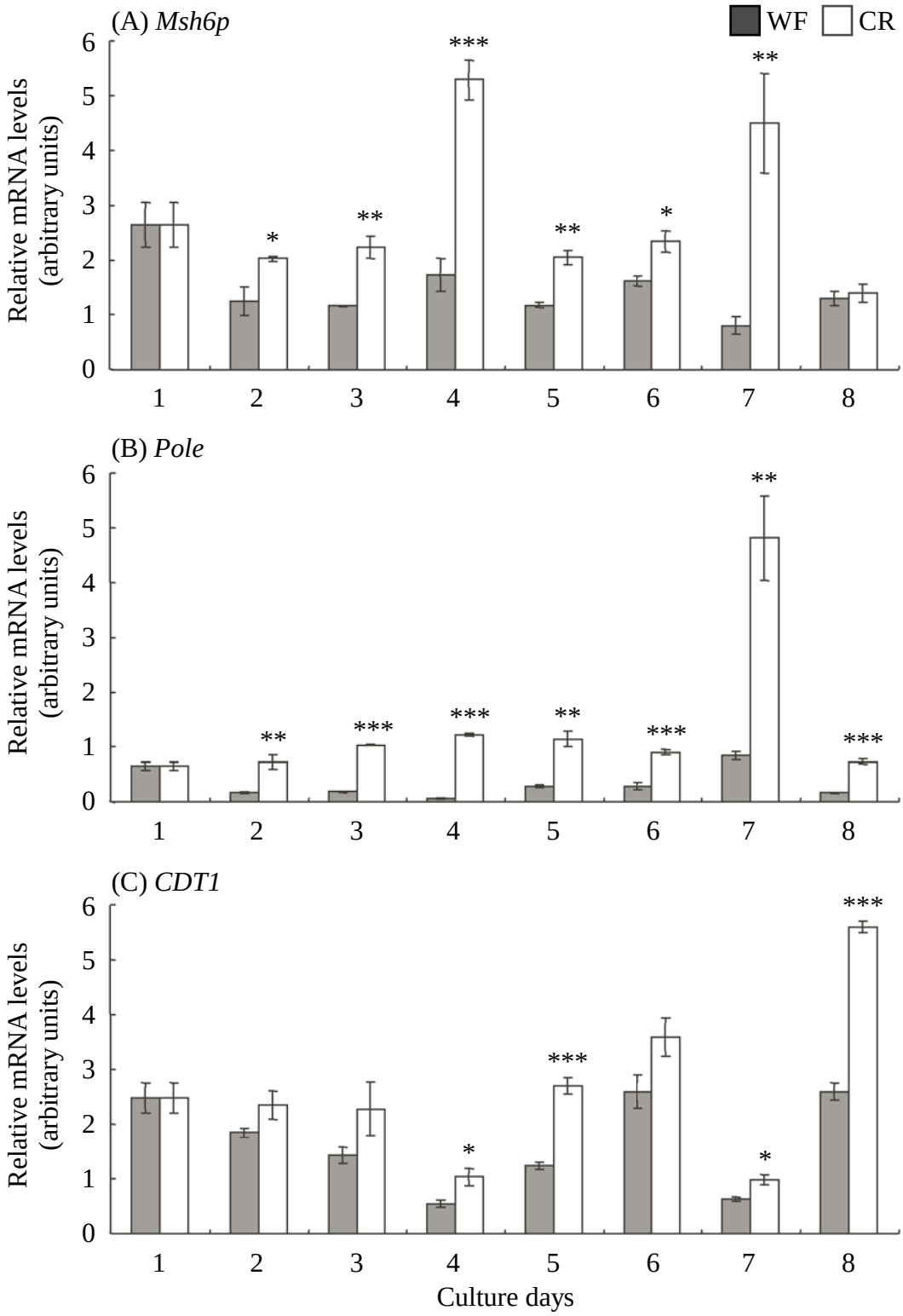


Fig. 2-2. The accumulated mRNA levels of 17 selected genes in the CR and WF rotifer populations of various ages. Thirty rotifers were collected from each group every day and used for total RNA extraction. First strand cDNA were diluted 100 times and the diluted cDNA were used for qRT-PCR. Relative mRNA levels of the target genes were determined by a comparative Ct method with reference to that of the elongation factor-1α gene (*EF-1α*) as the internal control. Data are mean ± s.e.m. (n=3) with significance levels of $p<0.05$ (*), $p<0.01$ (**), and $p<0.001$ (***).

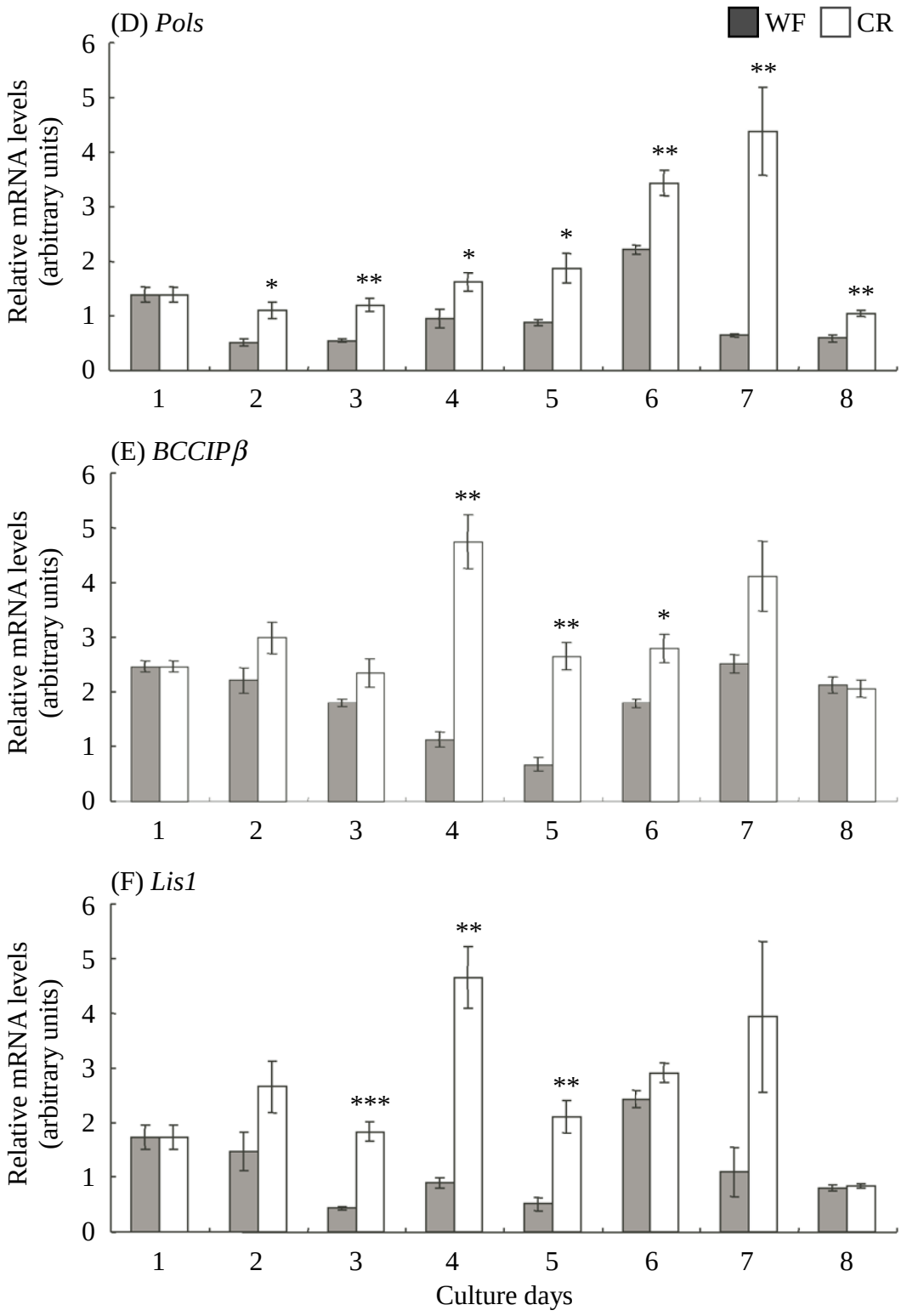
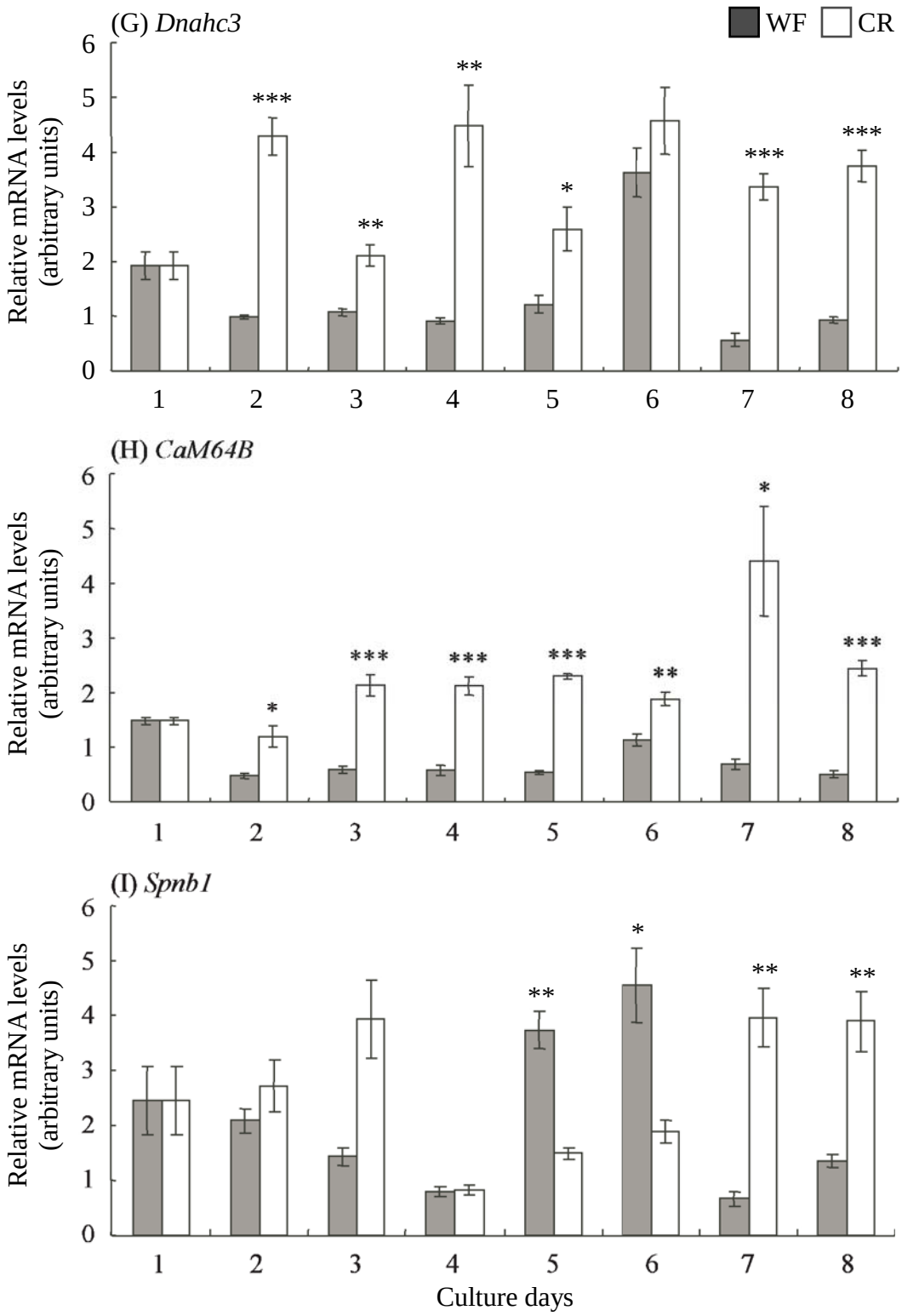


Fig. 2-2. (*Continued*)



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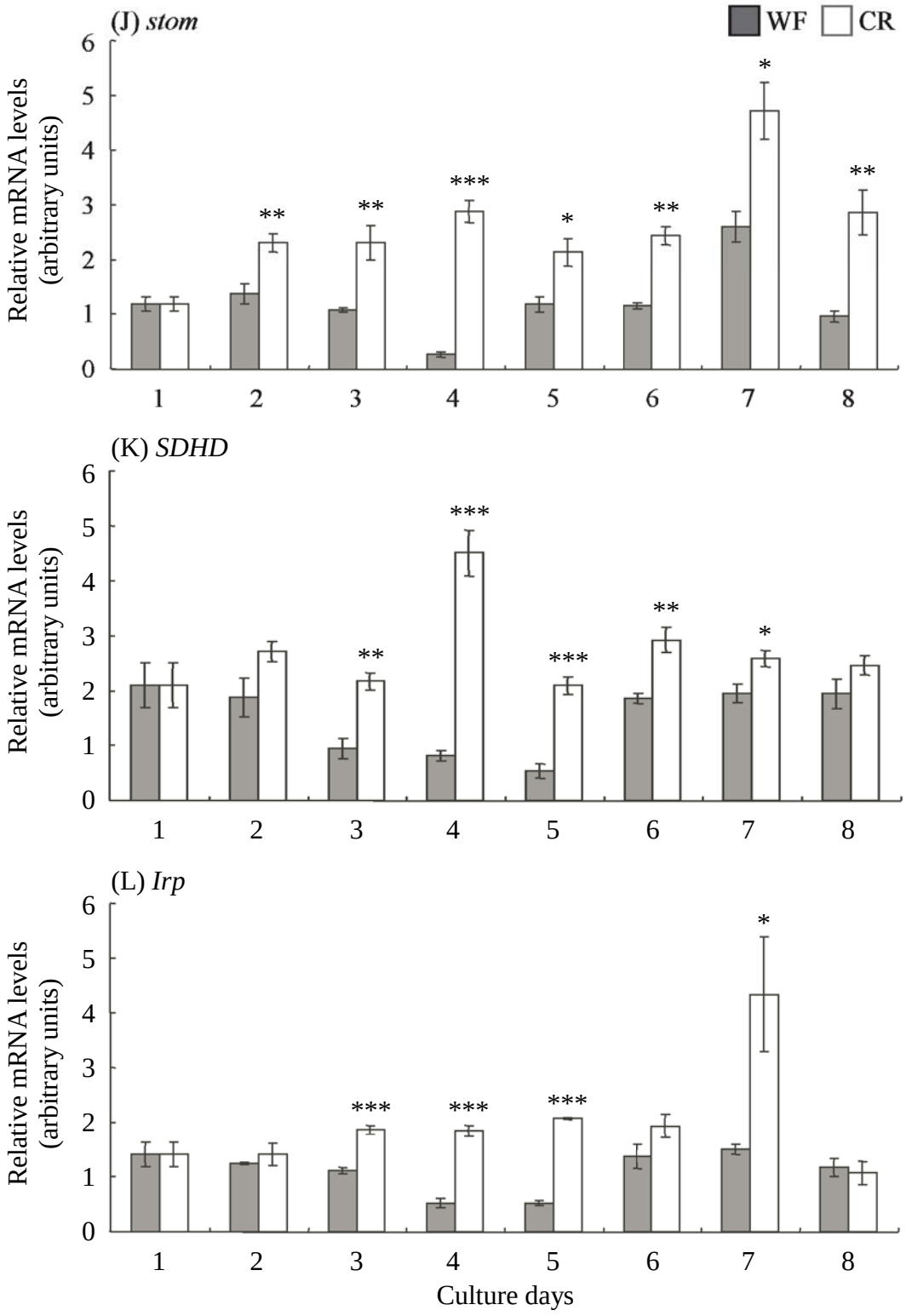

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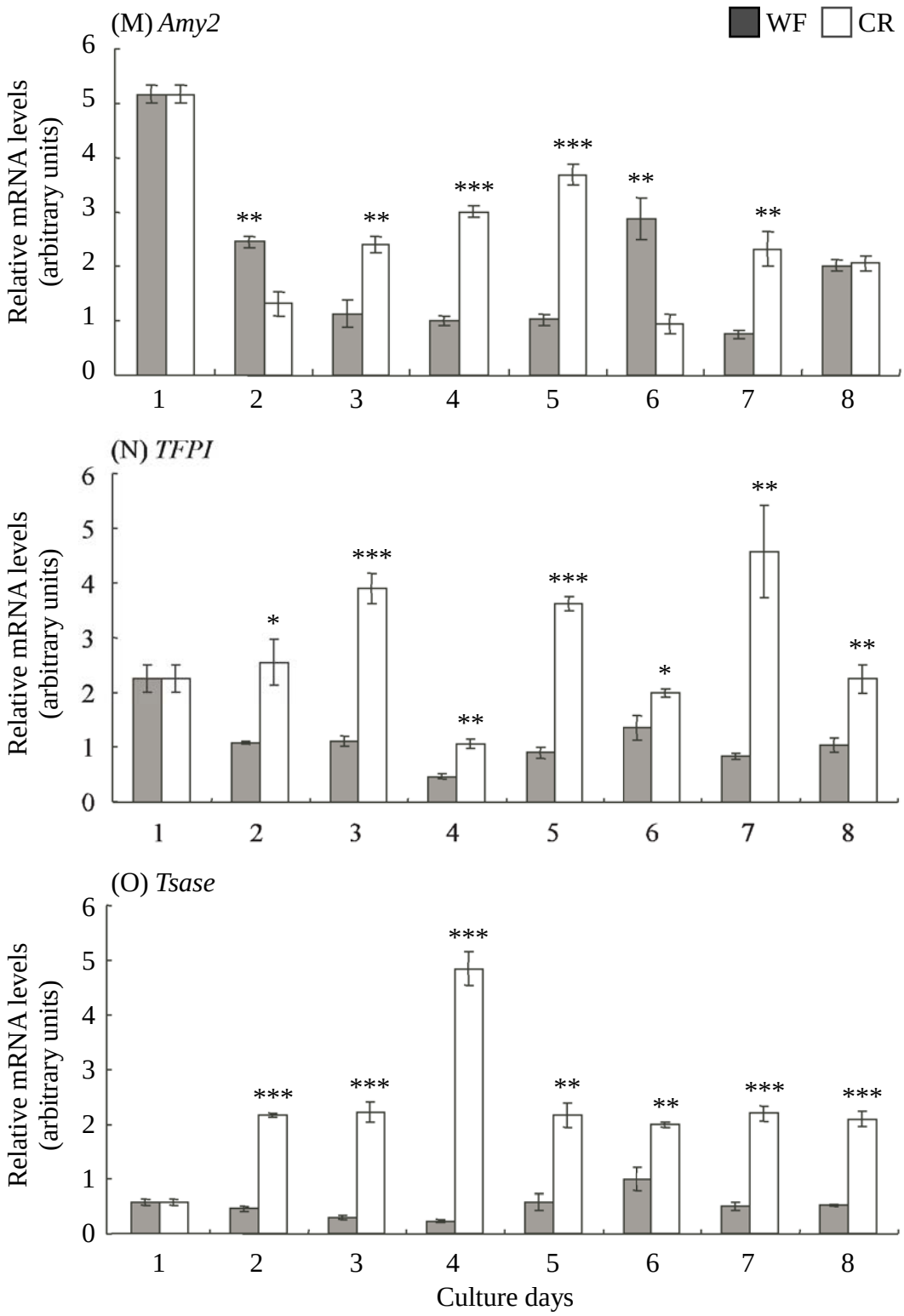

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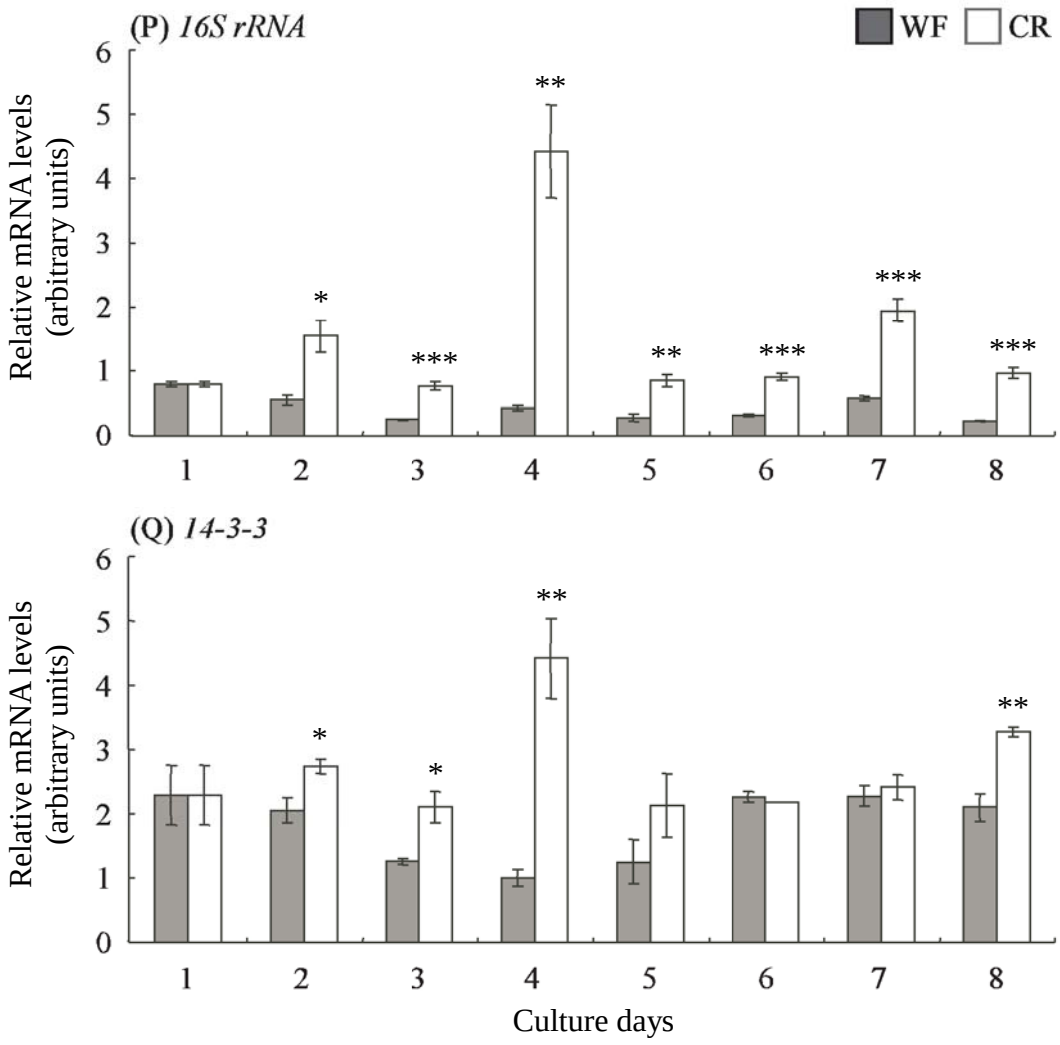

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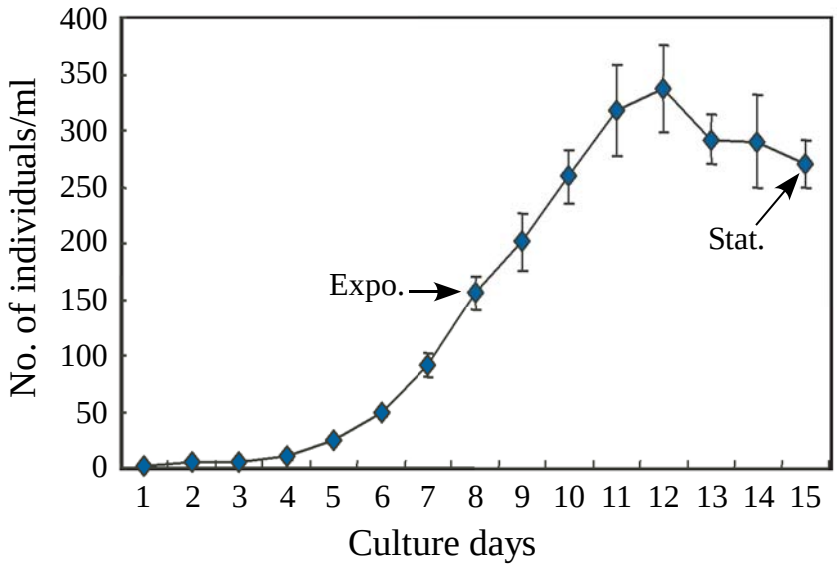


Fig. 2-3. Population growth and sampling points for the exponential (Expo.) and stationary (Stat.) phases. The mRNA levels of selected genes were determined in the exponential and stationary phases. The rotifers were reared in 10 beakers of a 100 ml volume, 5 each for the exponential and stationary phases. All cultures were began with 100 individual rotifers having almost the same size. The daily food amounts given to each culture were kept at a constant level. The old media were changed every day with fresh ones at the time of feeding. The numbers of individuals were counted daily by collecting a 1 ml of medium from each culture. Data are shown with mean ± s.e.m. (n=5).

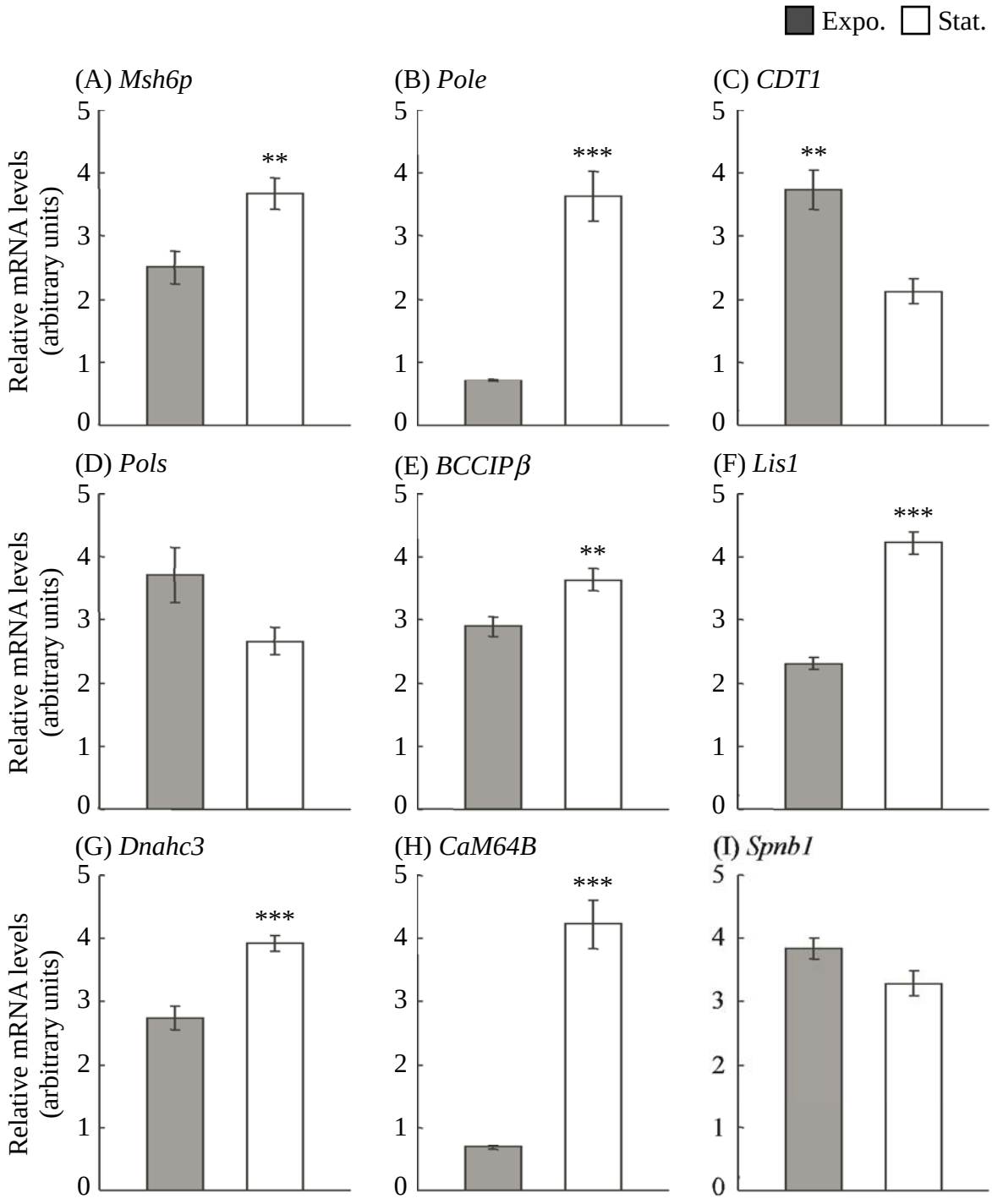


Fig. 2-4. The accumulated mRNA levels of the selected genes in the exponential (Expo.) and stationary phases of population growth. The mRNA levels of the selected genes were examined by qRT-PCR using gene-specific primers. Relative mRNA levels of the target genes were determined by a comparative Ct method with reference to that of the elongation factor-1α gene (*EF-1α*) as the internal control. Data are mean ± s. e. m. (n=3). Student's *t*-test (two-tailed) was employed for statistical analyses with significance levels of $p<0.01$ (**) and $p<0.001$ (***).

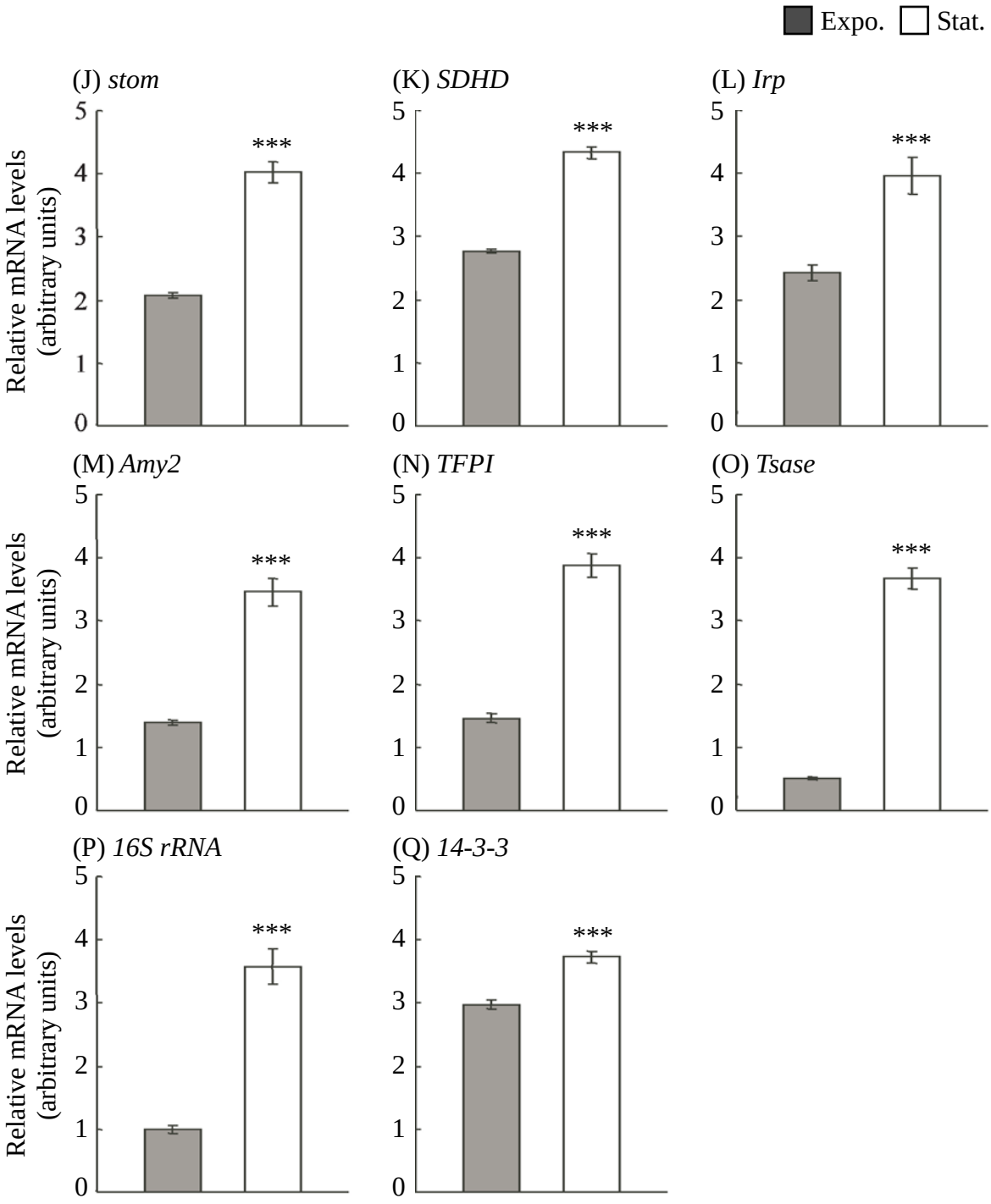


Fig. 2-4. (*Continued*)

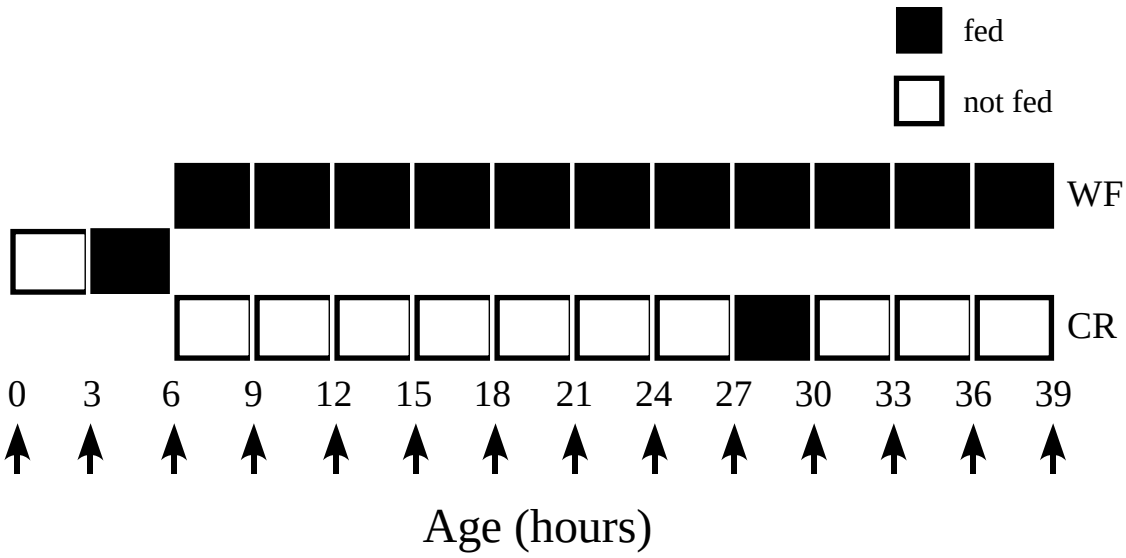


Fig. 3-1. Schematic diagram of animal culture and sampling for both BrdU labeling and quantitative real-time RT-PCR analyses. DNA synthesis and the mRNA levels of selected genes were studied in the calorie-restricted (CR) and well-fed (WF) rotifer population having almost the same ages. The shed eggs from the stock cultures were collected and hatched out. Neonates of 3 hour-post-hatching (hph) stage were transferred into a new medium and started feeding. After the first 3-hour-initial feeding period, the rotifers were divided into the CR and WF groups. The rotifers in the CR group were fed 3 h/day, whereas those in the WF control group were fed *ad libitum*. Sampling was conducted every three hours including 0 hph (egg) stage. Sampling points are indicated by arrows. For BrdU labelling, the samples were taken from 3 to 39 hph stages, whereas for qRT-PCR analyses, the samples were collected from 0 to 33 hph stages. Reproduction first appeared at 30 hph stage in the WF group.

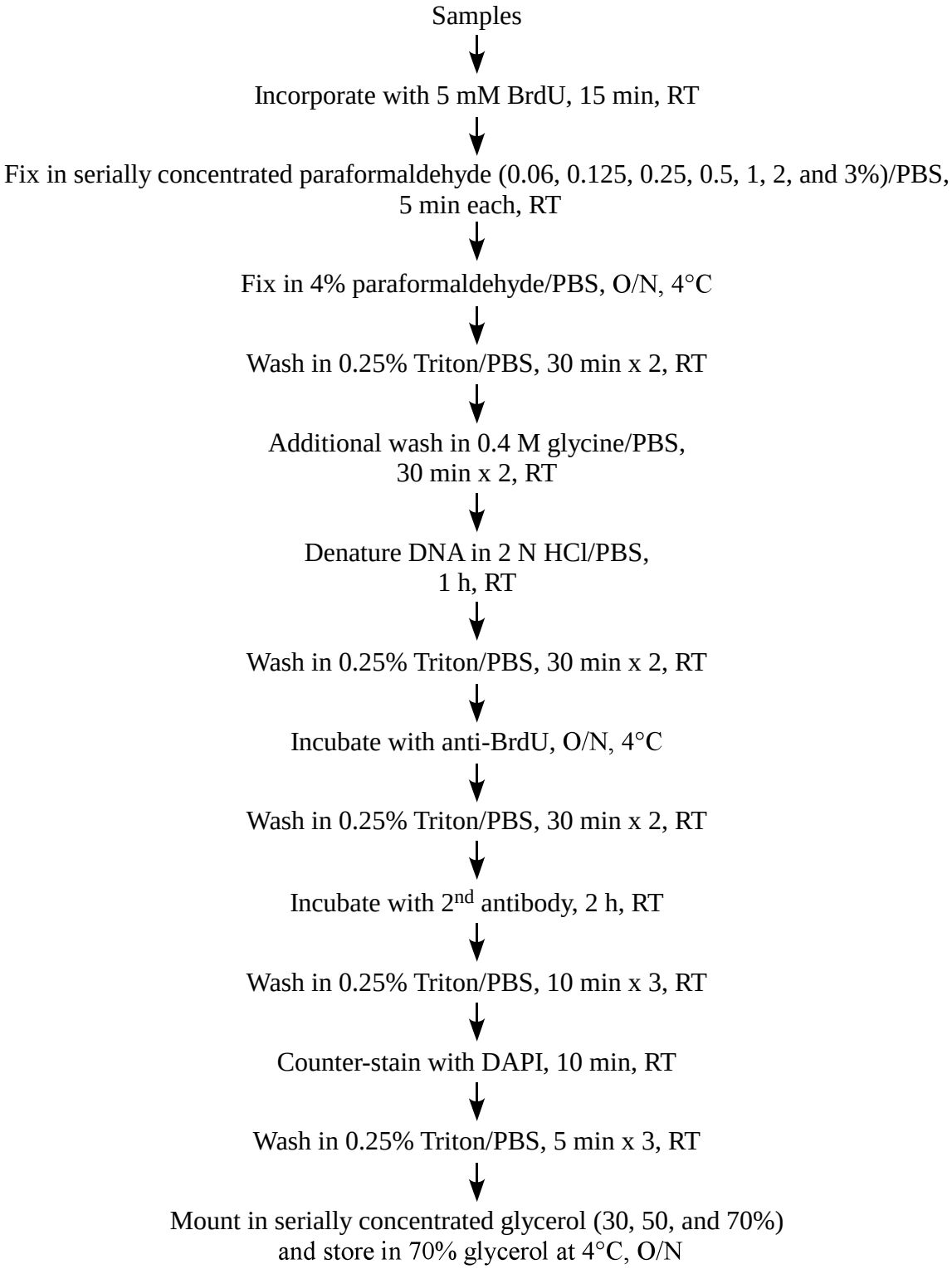


Fig. 3-2. Outline of BrdU labeling used for detecting DNA synthesized in the rotifer *Brachionus plicatilis*. Samples were infused with 5 mM BrdU. DNAs incorporated with BrdU were denatured with 2 N HCl and incubated with anti-BrdU antibodies. The samples were counter-stained with DAPI, and the BrdU signals were raised by incubating with the 2nd antibodies conjugated with an Alexa 555 fluorescent dye. RT, room temperature; PBS, phosphate-buffered saline; O/N, overnight.

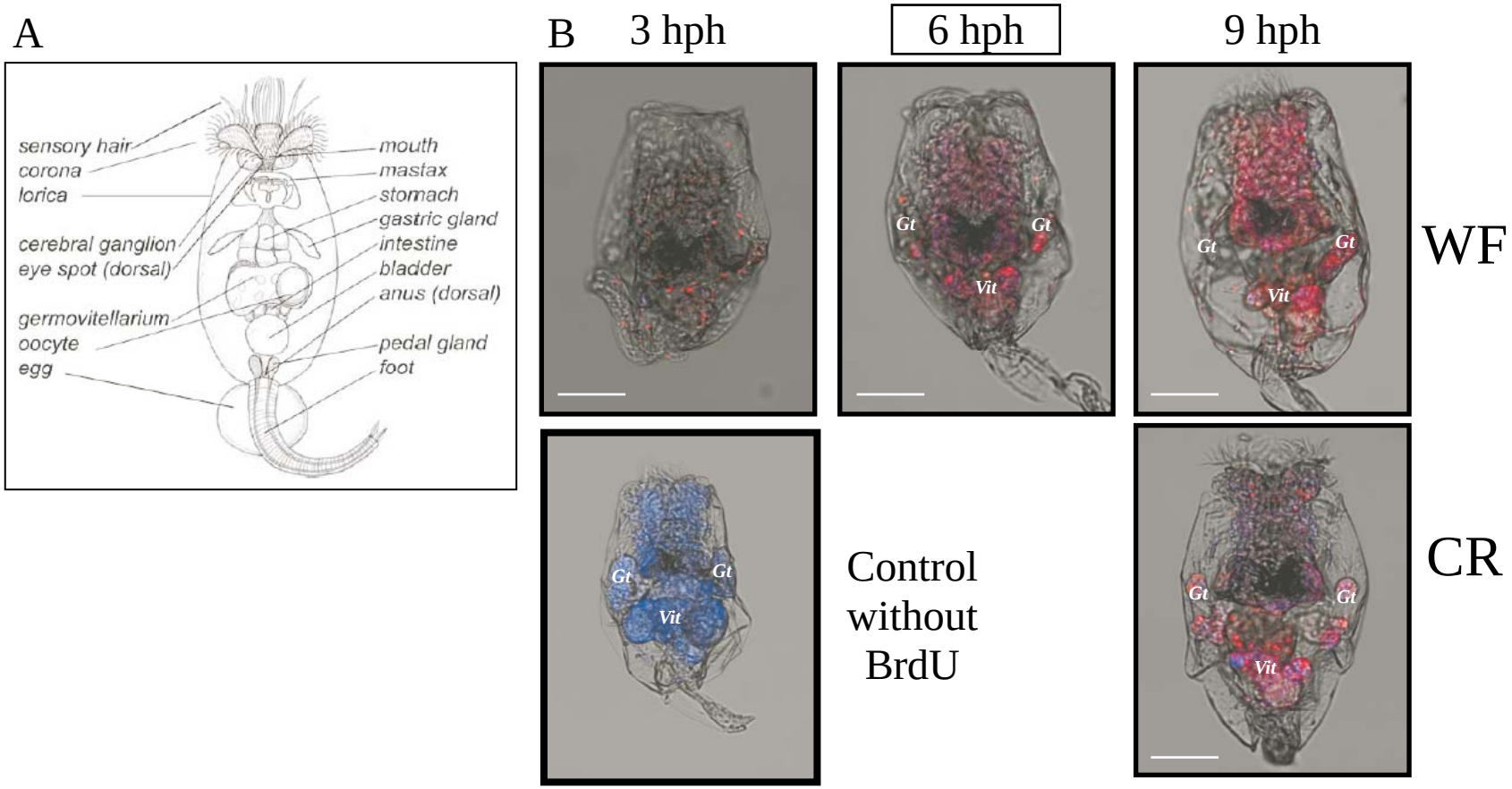
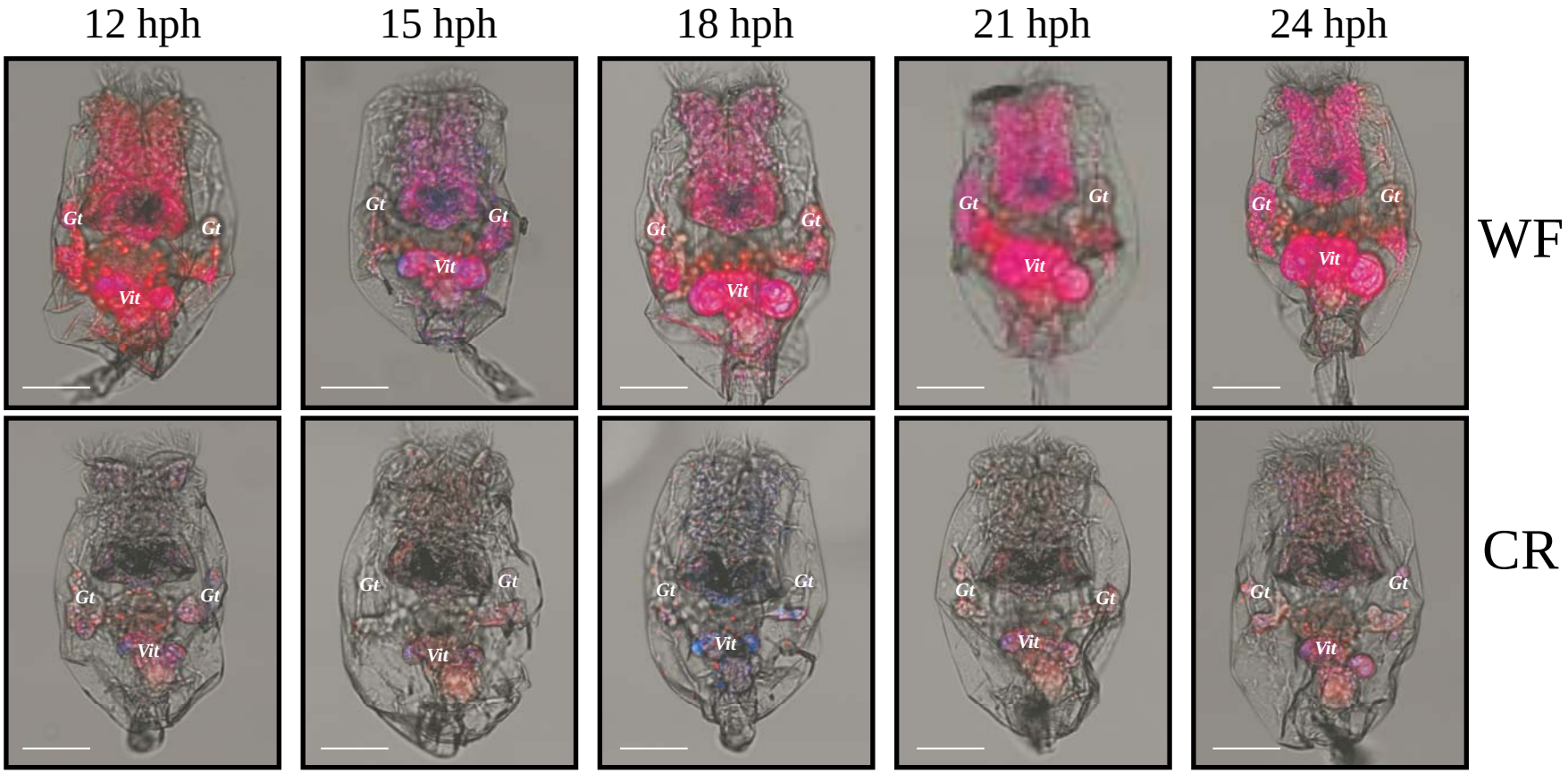


Fig. 3-3. BrdU labeling in the calorie-restricted (CR) and well-fed (WF) rotifers. DNA syntheses in the CR and WF rotifers of the same ages were detected by BrdU labeling. Samples were taken from 3 hour-post-hatching (hph) to 39 hph stages (B). Feeding periods in the CR group are boxed above the panels. A schematic drawing of *Brachionus plicatilis* is shown as an anatomical reference (A, Boell and Bucher 2008). Samples incorporated with 5 mM BrdU were incubated with anti-BrdU antibodies. The signals were raised by incubating with Alexa-fluo 555-conjugated 2nd antibodies (red). The samples were counter-stained with DAPI (blue). The control samples were not incorporated with BrdU, but were treated as the same procedures used for the experimental samples. *Eg*, egg; *Gt*, gastric gland; *Oo*, oocyte; *Vit*, germovitellarium or vitellarium. All figures are shown in ventral views. Scale bars are 50 μm in length.



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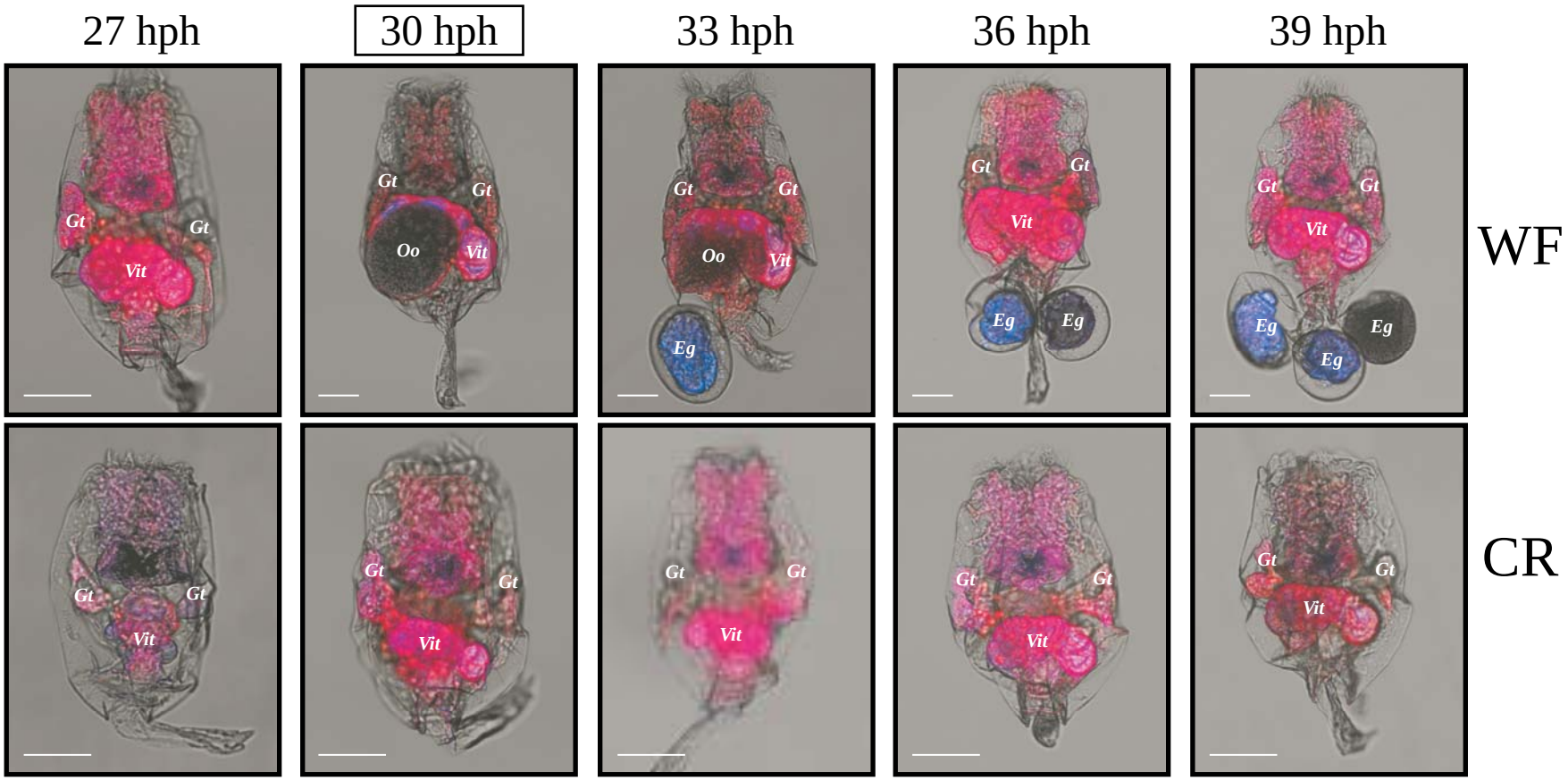

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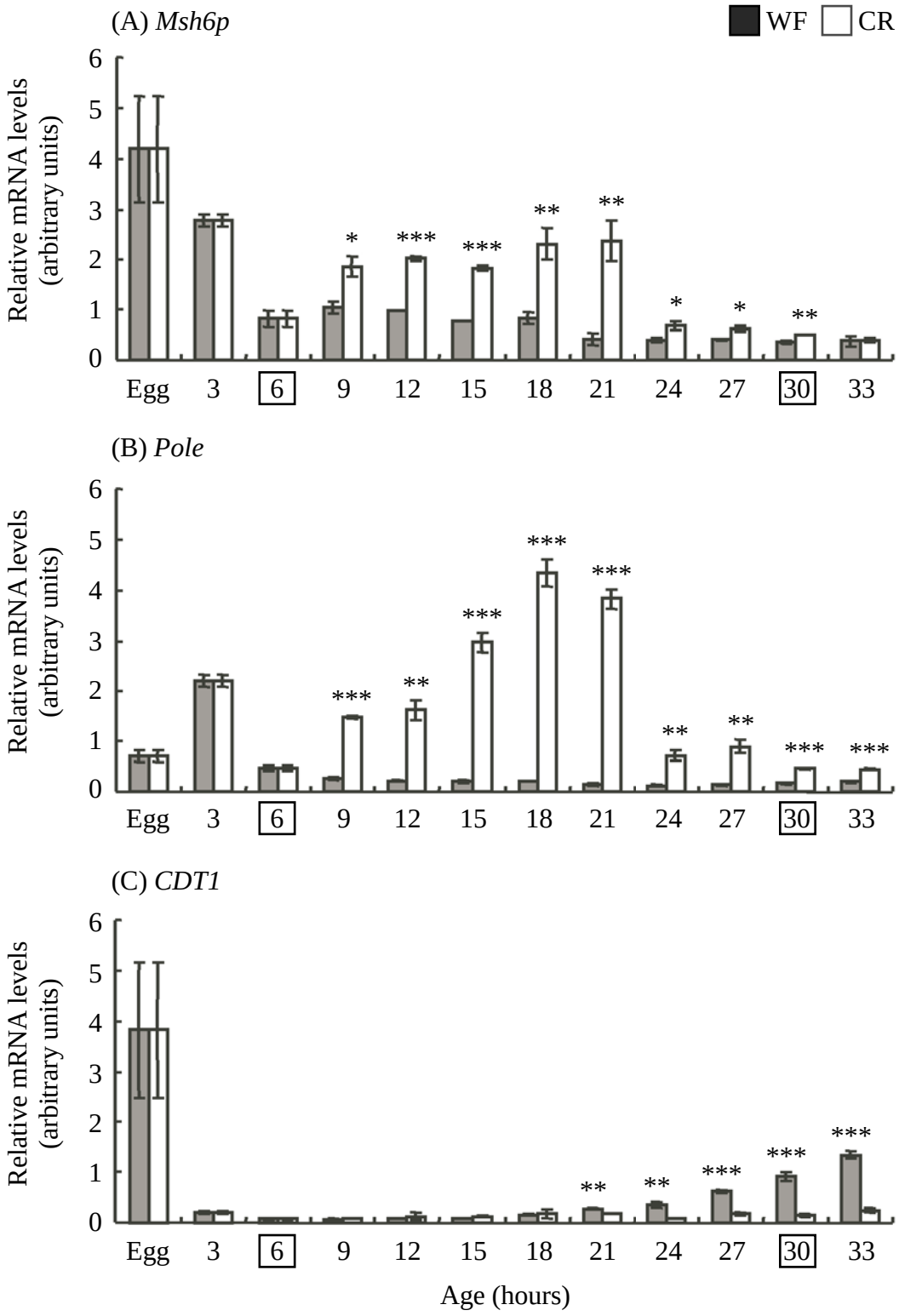
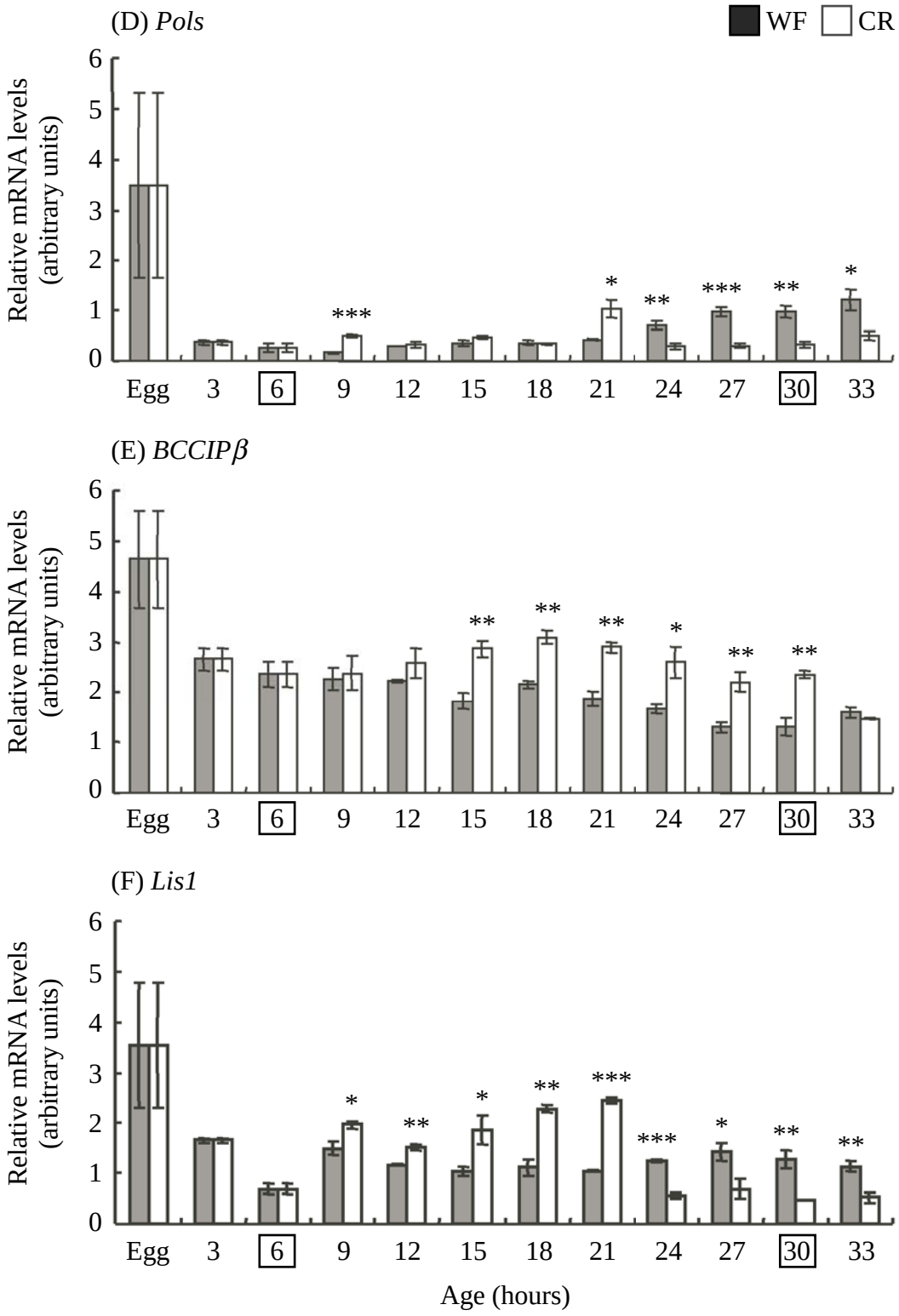


Fig. 3-4. The accumulated mRNA levels of 17 selected genes in eggs as well as in the CR and WF groups of the same ages. Thirty eggs and 30 rotifers were collected from each group and used for total RNA extraction. First strand cDNA were diluted 100 times and the diluted cDNA were used for qRT-PCR. Relative mRNA levels of the target genes were determined by a comparative Ct method with reference to that of the elongation factor-1α gene (*EF-1α*) as the internal control. Numbers boxed indicate three-hour-feeding periods in the CR group. Data are mean ± s.e.m. (n=3) with significance levels of $p<0.05$ (*), $p<0.01$ (**), and $p<0.001$ (***).



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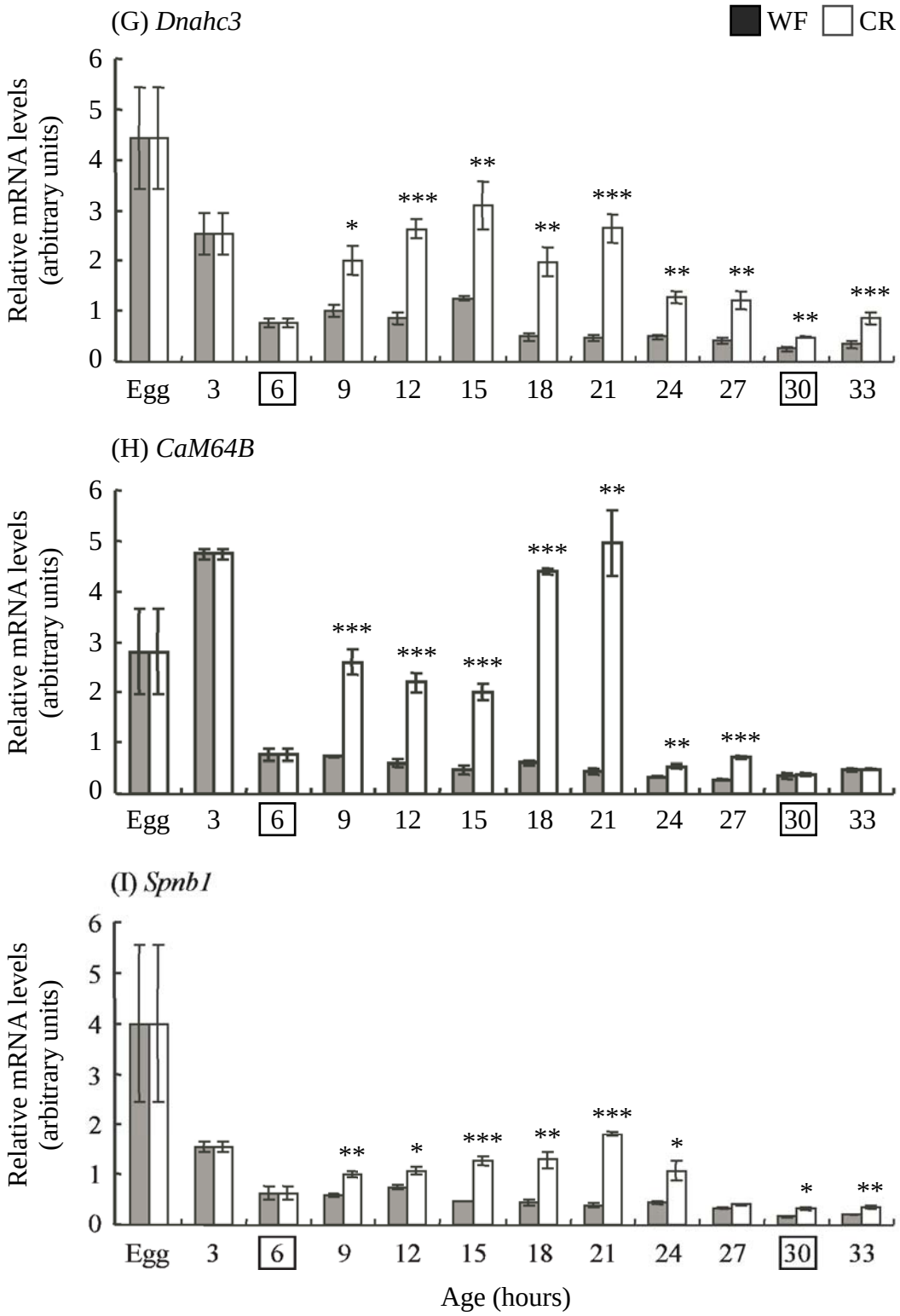

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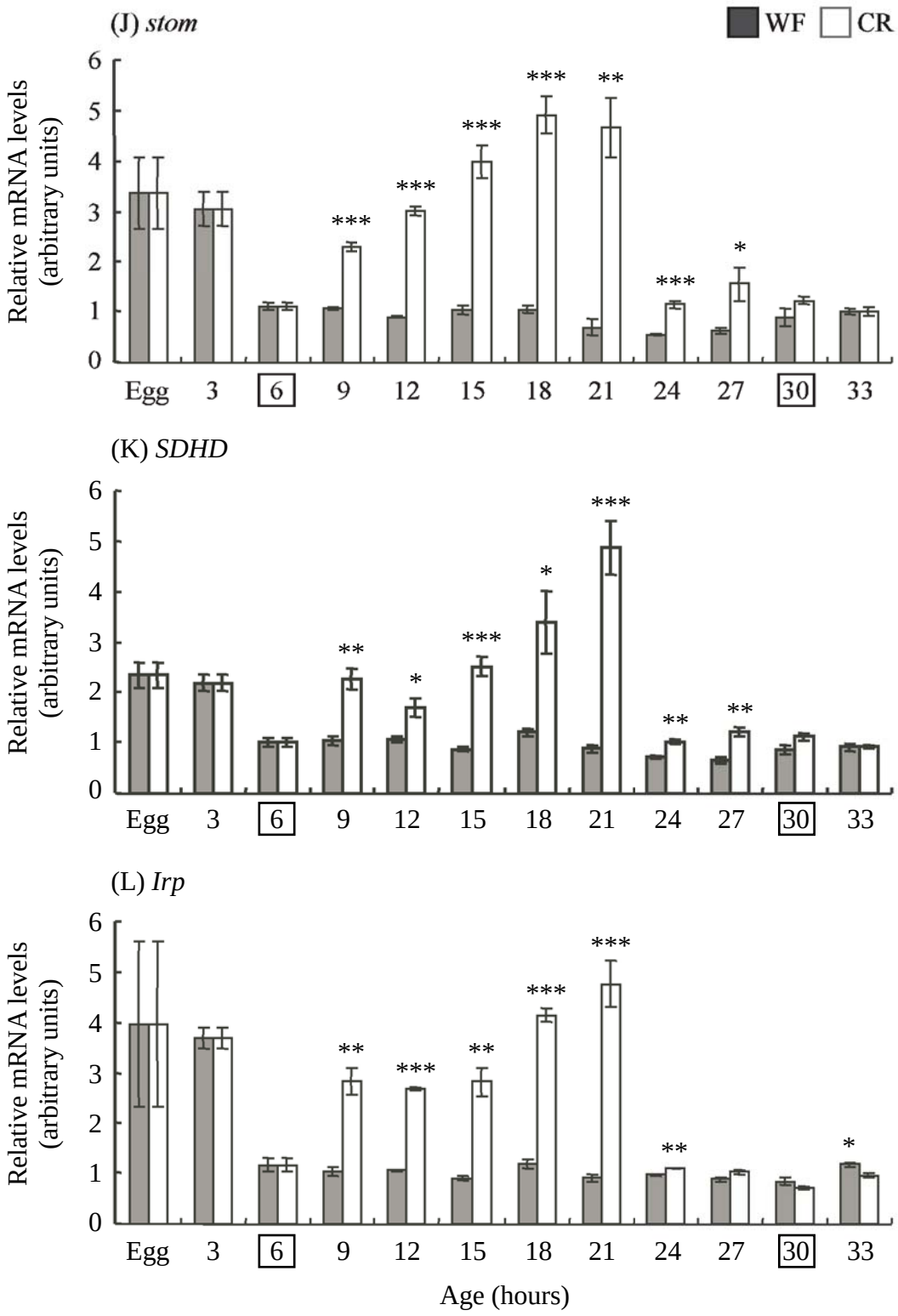

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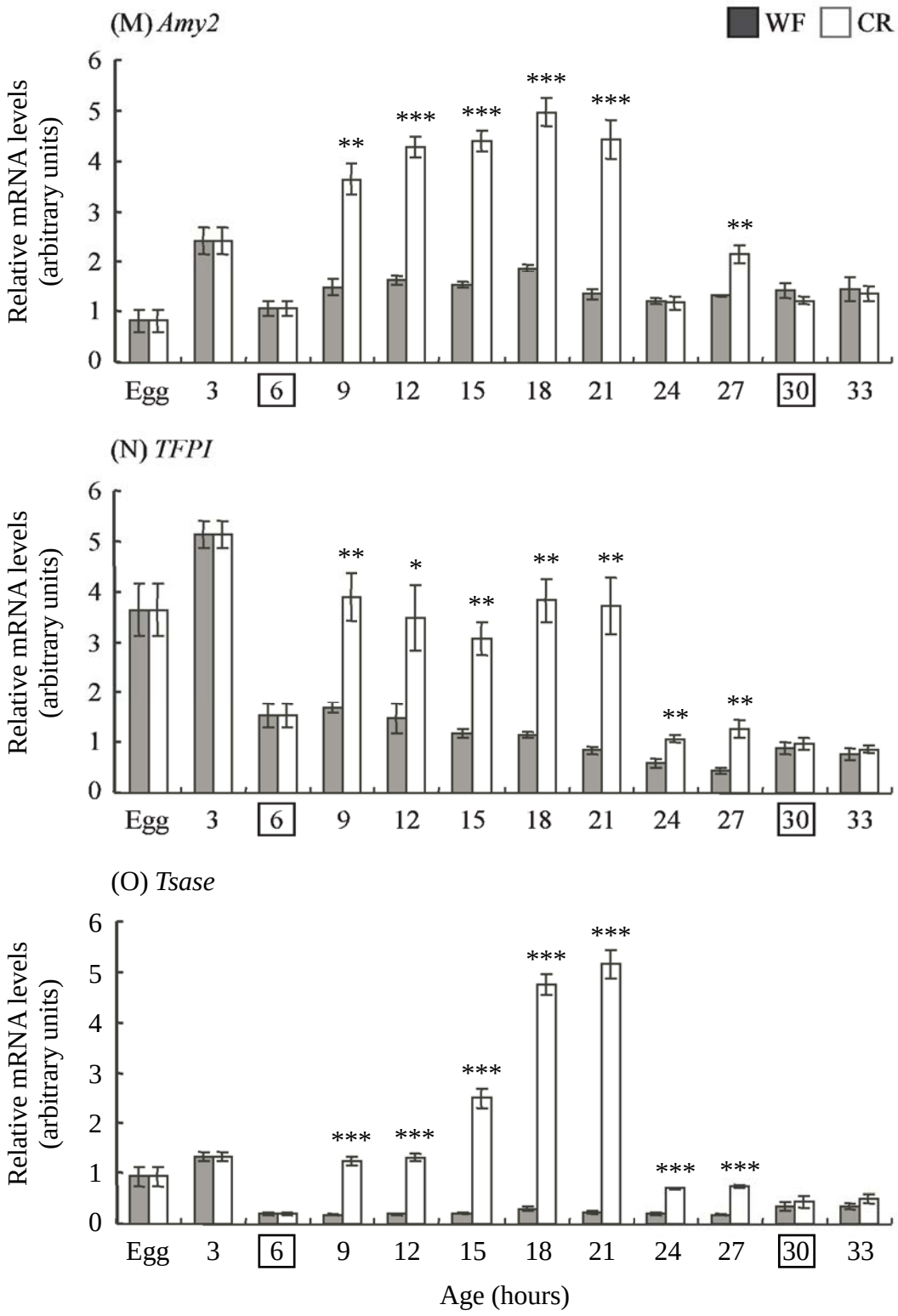

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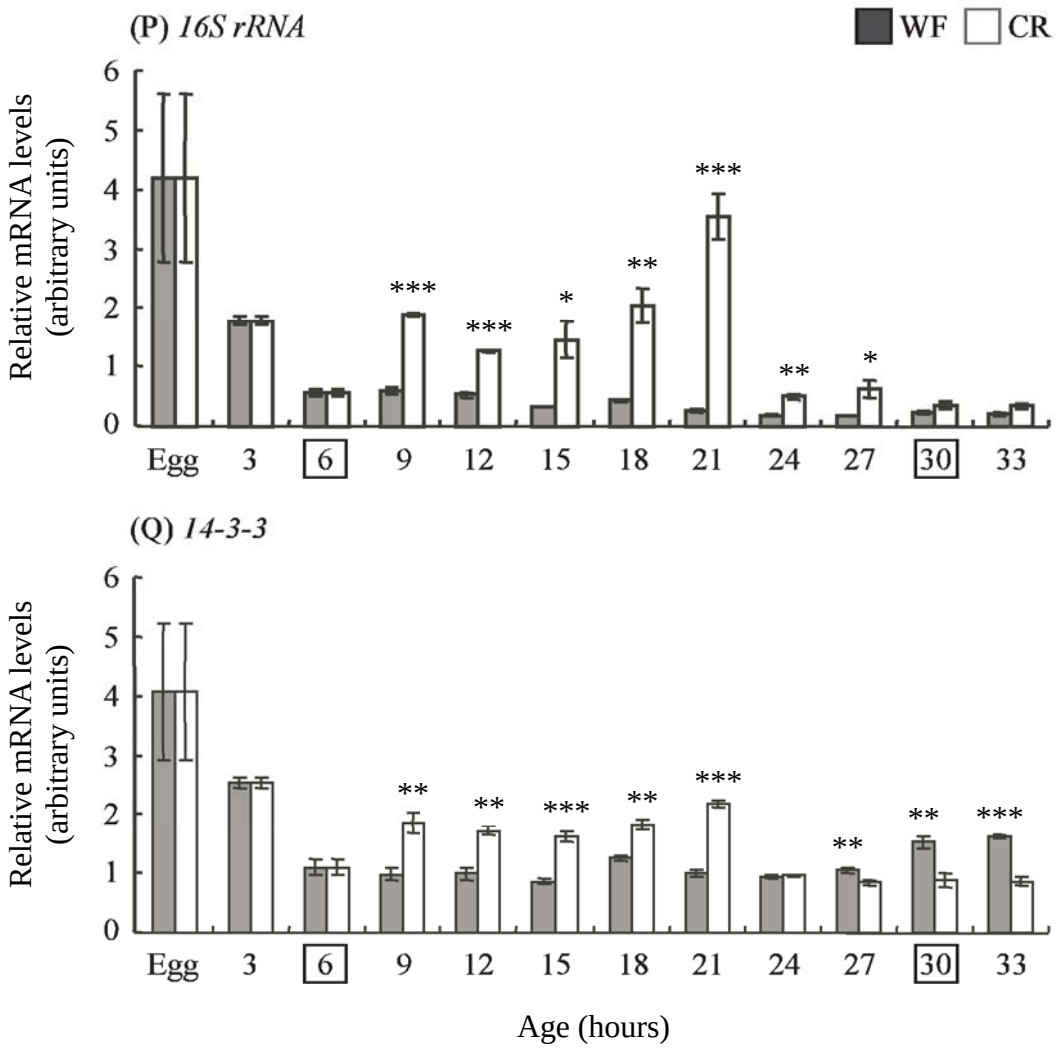

Fig. 3-4. (*Continued*)