VOLUME 1

Psychoneuroimmunology

Fourth Edition

Edited by Robert Ader

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Psychoneuroimmunology

FOURTH EDITION

Volume I

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Psychoneuroimmunology

FOURTH EDITION

Volume I

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Preface to the Fourth Edition

Psychoneuroimmunology is a convergence of disciplines—namely, the behavioral sciences, the neurosciences, endocrinology, and immunology—intended to achieve a more complete understanding of the way the interactions among these systems serve homeostatic ends and influence health and disease. As I have previously remarked, psychoneuroimmunology is an interdisciplinary field that has developed and now prospers by exploring and tilling fertile territories secreted by the arbitrary and illusory boundaries of the biomedical sciences. Disciplinary boundaries, codified by bureaucracies, are historical fictions that can restrict the imagination and impede the transfer and application of technologies and lend credence to Werner Heisenberg's assertion that "What we observe is not nature itself, but nature exposed to our method of questioning." Today, still, the business of science, by which I mean the training of scientists, the formulation of questions and hypotheses, the implementation of research, the communication among scientists, and even the funding of research, takes place within disciplinary boundaries that represent the disassembled parts of natural phenomena. This is not a representation of nature. It is an expedience that reflects our own intellectual limitations.

This theme of disciplinary integration is now being endorsed by the National Institutes of Health (NIH). The NIH Roadmap, a description of new trans-NIH initiatives, argues that, "Biomedical research traditionally has been organized . . . into broad areas of scientific interest and then [grouped] into distinct, departmentally based specialties. But, as science has advanced over the past decade . . . , two fundamental themes are apparent: the study of human biology and behavior is a wonderfully dynamic process, and the traditional divisions within biomedical research may in some instances impede the pace of scientific discovery. To lower these artificial organizational barriers, the NIH will implement several initiatives designed to facilitate interdisciplinary research collaborations and research training and, ultimately, lead to the development of new hybrid disciplines that will provide a more complete understanding of psychological, social and biological interactions in health and disease." Psychoneuroimmunology is one such hybrid discipline that should prosper under these NIH initiatives. This is the temporal context in which I proposed a fourth edition of *Psychoneuroimmunology*.

Like previous editions, *Psychoneuroimmunology-IV* is addressed to a broad audience in a continuing effort to draw attention to the field and promote interdisciplinary research in the laboratory and in the clinic. The primary targets would be laboratory and clinical investigators in endocrinology, immunology, neurochemistry, neurophysiology, pharmacology, psychiatry, psychology, virology, and medical specialists in allergic and infectious diseases, oncology, rheumatology, etc. In contrast to previous editions, the editorial responsibilities for *Psychoneuroimmunology-IV* were shared among a board of Associate Editors. These individuals, Robert Dantzer, Ronald Glaser, Cobi Heijnen, Michael Irwin, David Padgett, and John Sheridan, are well known and respected investigators who did a superb job in selecting and editing the chapters in their respective parts.

Having established that (a) lymphocytes bear receptors for neurotransmitters, neuropeptides, and hormones and that activated lymphocytes can produce these same neuroendocrine signals, and that (b) neurons bear receptors for messenger molecules produced by cells of the immune system and that these cytokines can be released by cells of the neuroendocrine system, current research on the mechanisms involved in this neuroendocrineimmune-circuitry are presented in Part I of this volume. Emphasizing either shared mediators or shared recep-

tors, these several chapters describe functional neuroendocrine-immune system regulatory pathways at the intercellular, interorgan, and whole body level—and how these circuits might influence health and disease. Continuing and reinforcing this discussion of shared mediators and receptors, Part II illustrates the diversity of effects and the complexity of the circuitry involved that has made research on cytokines and neuroinflammatory processes a rapidly expanding interdisciplinary area of research in the neurosciences, including behavior.

Part III details the influence of behaviors, disordered behavioral states, and behavioral interventions, including classical conditioning, on immune functions that bear on health. The premise underlying this research is that psychosocial and stressful life events induce emotional responses that are associated with neuroendocrine and autonomic nervous system changes capable of modulating immune function and, thus, the susceptibility and/or progression of disease. It is further hypothesized that interventions calculated to elicit positive emotions and behaviors might, through alterations in immune states, ameliorate the effects of stressful life experiences on disease.

The final two Parts most directly address the perennial issue of whether stress-induced changes in immune function are sufficiently large (and/or of sufficient duration) to influence the health of the organism. Within the context of behavioral medicine or psychosomatic medicine, could changes in immune function serve as mediators of the effects of psychosocial factors, including stressful life experiences, on health and disease? The several chapters in Part IV on stress and immunity affirm, within the immune system, an existing literature indicating that different stressors elicit different physiological responses and, therefore, would have different effects on different disease processes. This part also reviews the effects of positive affect and social relationships on immune function and their possible health consequences; the data on the role of stressful life experiences and associated changes in immune function in allergic diseases, wound healing, reactivation of latent viruses, the progression of malignant disease, and how stressful life experiences are capable of altering the individual's response to vaccines. Part V provides further examples of how the immune system plays a role in mediating the effects of psychosocial factors on specific disease processes. These chapters review the provocative new studies of immune system involvement in cardiovascular disease, skin disorders, obesity, infectious and autoimmune diseases—and the psychoneuroimmunologic mechanisms that may play a role in host resistance.

Although the nervous, endocrine, and immune systems evolved to serve specialized functions, the premise underlying psychoneuroimmunology, confirmed by the extraordinary amount of research that has been conducted in the past couple of decades, is that each of these "systems" is also capable of responding to information derived from the other. Together, behavioral, neural, endocrine, and immune processes of adaptation constitute an integrated network of defenses and, insofar as immunoregulatory processes are concerned, the assumption of an autonomous immune system is no longer tenable. It is not possible to obtain a full understanding of immunoregulatory processes without considering the organism and the internal and external environment in which immune responses take place.

The first edition of *Psychoneuroimmunology* was published 25 years ago. It described the relatively meager amount of research being conducted on the relationship between the brain and the immune system. However, it was, as one reviewer prophesied, the signature volume of a new field of research. That new field of research has since come of age and is being described as a paradigm shift in the way we view the multi-tiered organization of adaptive processes. Certainly, that is not a universally accepted proposition. Psychoneuroimmunology has been and, in reduced volume, continues to be assailed by gratuitous and uninformed commentaries. That is not altogether surprising. Any number of writers has commented that the everyday business of most scientists does not concern itself with the development or implementation of new ideas and theories—and is often intolerant of such developments by others. "Normal science," as Thomas Kuhn refers to it in *The Structure of Scientific Revolutions* (University of Chicago Press, 1970), is concerned with an elaboration of the phenomena that the prevailing paradigm already supplies. And, in accord with the prevailing paradigm, we refer to *connections between the CNS and immune system*, which is not an especially satisfactory or accurate expression. It does not adequately describe the essence of the integration that the term *psychoneuroimmunology* is meant to convey. The phrase enables one to retain the historical fiction that these are separate and distinct systems; it is a two-dimensional expression for a three-dimensional concept.

Psychoneuroimmunology does magnify the complexity of already complex fields of study. That, however, is a small price to pay for a more complete understanding of biological processes that constitute the foundation of the study of health and illness. As we learn more about how behavioral, neural, and endocrine states influence immunologically based dis-

eases and how immune processes affect behavior and what are presumed to be endocrine or neurologic disorders, there may be a need to redefine the nature of some diseases and, thus, the strategies for therapeutic interventions. Accepting the notion that there is a single, integrated system of adaptive processes presents new challenges and new opportunities for approaching the study of health and the treatment and prevention of disease. The authors of *Psychoneuroimmunology-IV* invite your participation in this venture.

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Dr. Robert Ader is Distinguished University Professor at the University of Rochester School of Medicine and Dentistry. He received his Ph.D. in psychology from Cornell University in 1957 and immediately thereafter joined the Rochester faculty, becoming Professor of Psychiatry and Psychology in 1968. He was awarded an honorary M.D. from Trondheim University in Norway in 1992 and an honorary D.Sc. from Tulane University in 2002. Dr. Ader was Visiting Professor at the Rudolf Magnus Institute for Pharmacology in Utrecht, The Netherlands (1970–71), and a Fellow at the Center for Advanced Study in the Behavioral Sciences at Stanford (1992–93). He serves on several Editorial Boards and was Editor-in-Chief of *Brain, Behavior and Immunity* from 1986 through 2002. Dr. Ader is a past President of the American Psychosomatic Society, the International Society for Developmental Psychobiology, the Academy of Behavioral Medicine Research, and was Founding President of the Psychoneuroimmunology Research Society.

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PROLOGUE

Exploring the Phylogenetic History of Neural-immune System Interactions: An Update

NICHOLAS COHEN AND KEVIN S. KINNEY

I. INTRODUCTION

Psychoneuroimmunology, the study of behaviorally associated immunological changes and immunologically associated behavioral changes that result from reciprocal interactions among the nervous, endocrine, and immune systems, has emerged as a new field of scientific inquiry within the past 2 decades (Ader, 1981; Ader et al., 1991, 2001). It is a field that has been defined phenomenologically and is currently being explored mechanistically, primarily by studying rodents and primates. Although hundreds of investigators are using these and other mammalian species to address basic and clinical facets of psychoneuroimmunology, we are aware of only a handful of laboratories in which invertebrates, avian, and ectothermic (cold-blooded) vertebrate species are serving, or have served, as living tools to probe the evolutionary origins of neural-immune system interactions. In 2001, we published a comprehensive review of the research from these laboratories (Cohen and Kinney, 2001); the present review updates that information. The reader should bear in mind that the descriptive comparative approach we have taken here allows us only to make educated guesses about the true evolutionary history of the integration of two complex physiological systems.

To avoid redundancy with what is presented in the rest of this 4th edition of *Psychoneuroimmunology*, we will not summarize the voluminous data from research with mammals as we did in our earlier review (Cohen and Kinney, 2001). The main phenomenology emerging from the literature that deals with neural-immune system interactions in rodents and primates, however, still serves as the gold standard for the questions asked by investigators using non-mammalian model systems. Thus, for comparative purposes, the mammalian reference points include the following facts: (1) mammalian lymphoid tissues are richly innervated (Felten et al., 1992; Felten et al., 2003); (2) cells of the mammalian immune system express receptors for neuropeptides, neurotransmitters, and hormones (Sanders et al., 1997, 2001); (3) activation of these receptors by their appropriate ligands affects functional behavior of the cells (Sanders and Straub, 2002; Sanders et al., 2001); (4) the SNS exerts a tonic regulatory role over the immune system as revealed, for example, by experiments

involving sympathectomy (Kruszewska et al., 1995, 1998); (5) cells of the immune system themselves produce, as well as respond to, neuropeptides and hormones (Blalock, 2005; Smith, 2003); (6) cytokines (e.g., IL-1, IL-6, TNF-α) produced by cells of both systems act as signal molecules in the bi-directional dialogue between the nervous and immune systems (Danzer et al., 2002; Goehler, et al., 1997; Maier, 2003; Maier and Watkins, 1998); and (7) behavioral responses to diverse stimuli (stressors) can trigger central neuroendocrine and peripheral autonomic responses that can alter immune parameters and, thereby, under certain conditions, affect the health of the organism (Glaser et al., 1999).

II. NEURAL-DEFENSE SYSTEM INTERACTIONS IN INVERTEBRATES

By all current definitions, invertebrates display features of innate immunity in the complete absence of adaptive immunity (e.g., major histocompatibility complex [MHC], immunoglobulin genes, re-arranging T-cell receptor genes, immunological specificity, and memory). In the past 15 years, two major research groups, one in Italy and the other in New York state, have explored the possibility that communication between the neuroendocrine and innate defense systems exists in invertebrates as well as vertebrates. These investigators have used four basic interrelated approaches to address this phylogenetically critical issue. In the following review of their work and the more recent work of others, the generic term *hemocyte* refers to the invertebrate equivalent of vertebrate blood leukocyte.

A. Synthesis of Neuroendocrine and Neurotransmitter Molecules by Invertebrate Hemocytes

The first approach focused on whether endogenous neuroendocrine and/or neurotransmitter substances are synthesized by, and can affect the behavior of, invertebrate blood cells. Hemocytes from several molluscan species (*Planorbarius corneus, Lymnaea stagnalis, Mytilus edulis*) exhibit immunoreactivity for several vertebrate neuropeptides including met-enkephalin, oxytocin, somatostatin, vasoactive intestinal peptide (VIP), substance P (SP) (Ottaviani and Cossarizza, 1990), ACTH (Ottaviani et al., 1991; Ottaviani et al., 1992a; Smith et al., 1991), and β-endorphin (Ottaviani et al., 1990). ACTH- and TNF-α–like molecules are also found in some types of leukocytes residing in the hemolymph of the dipteran *Calliphora vomitoria;* staining for both ACTH and TNF-α of the mitotically active plasmacytes was related to their activated state during the formation of capsules to wall off foreign substances (Franchini et al., 1996b). Immunoreactive met-enkephalin has also been detected in the coelomic fluid of earthworms, and treatment of earthworm coelomocytes with DAMA stimulates coelomocyte migration, much as is seen in human granulocytes and molluscan hemocytes (Cooper et al., 1993). Eleven years ago, Ottaviani et al. (1995a) reported that hemocytes from freshwater snails, *Planorbarius corneus* and *Viviparus ater,* express pro-opiomelanocortin (POMC) mRNA as assessed by *in situ* hybridization with a digoxigenin-labeled human DNA probe. Interestingly, this probe did not detect POMC mRNA in another morphologically distinct hemocyte from these species, a hemocyte that Ottaviani (1992) believes has features of the vertebrate T-cell. This is the same probe mentioned subsequently in connection with detection of POMC mRNA in phagocytic leukocytes from both the edible frog and goldfish, and in lymphocytes from frogs but not fish (Ottaviani et al., 1995a).

It also appears that "stress" stimulates invertebrate hemocytes to produce endogenous neural-immune mediators. For example, Stefano et al. (1989b) found elevated levels of endogenous morphine-like material in the hemolymph of *Mytilus* that had been subjected to electrical shock combined with mechanically preventing closure of their shells. Concurrent with this rise was a substantial increase in the proportion of activated (ameboid, as opposed to rounded or resting) hemocytes (Stefano et al., 1993). With respect to stressors and immunological changes in invertebrates, it is worth noting that Malham et al. (2003) reported that abalone subjected to 15 minutes of mechanical disturbance (i.e., shaking in a rotating box) resulted in an elevation of norepinephrine (NE) and epinephrine (EPI) levels. Whether this increase was causally responsible for the accompanying reduction of numbers of circulating hemocytes, their migratory and phagocytic activity, and respiratory burst is possible, but this component of the regulatory pathway has yet to be studied. Interestingly, these changes in immune parameters were short lived in that the values returned to baseline levels (with the exception of superoxide anion production) 100–480 minutes after stress exposure. In a related study, Malham and co-workers (2002) "stressed" octopuses (*Eledone cirrhosa*) by handling them and exposing them to air for a few minutes. They found that both NE and EPI were released into hemolymph but that the increased levels returned to basal levels within 30–60 minutes after stressor exposure. They also saw a decrease in the numbers of circulating hemocytes that was followed by a rebound effect (i.e., greater numbers than controls within an hour). In contrast to

their observations with abalone, the stressor in the octopus effected an increase in hemocyte activity (measured by phagocytosis of heat-killed *Vibrio*) that peaked at 1 hour and returned to baseline by 2 hours. The production of superoxide anions peaked as early as 5 minutes post stressor exposure and remained elevated for upwards of 2 hours. The norendocrine effectors responsible for these transitory changes are unknown, as is whether such short-lived changes have repercussions with respect to the health of these invertebrate species. There is, however, a very recent study that addresses the long-term health consequences of a stressor exposure in another invertebrate, the fresh water clam *Anodonta piscinalis* (Saarinen and Taskinen, 2005). These investigators explored the susceptibility of *Anodonta* to the ergasilid copepod, *Paraergasilus rylovi.* Clams were field collected from two populations in late summer. They were then transported to the laboratory and marked. The stressed clams were subjected to low oxygen for 25 days, whereas the unstressed control clams were housed in their lakes of origin for the same period. Eleven months after exposure to the stressor, the stressed clams were more intensively parasitized than controls. They also showed lower growth, lower reproduction, and poorer survival than the "unstressed" control clams. Thus, this model suggests that even in an invertebrate, a stressor may evoke long-lasting effects on susceptibility of natural populations to parasitism.

B. Effects of Mammalian Neuroendocrine and Neurotransmitter Molecules on Invertebrate Hemocytes

Several investigators have examined the possible influence of exogenous neuroendocrine, neuropeptide, and/or neurotransmitter messenger molecules on the behavior of invertebrate hemocytes. Recent research on crustaceans by Cheng and colleagues (2005) revealed that 4 hours after white shrimp received 10^{-7} M dopamine, there was a 25% decrease in total hemocyte count, a 15% decrease in phenoloxidase activity, a 21% decrease in respiratory burst, and a 50% decrease in superoxide dismutase activity. Further, the phagocytic activity and clearance of *Vibrio* also diminished significantly, and bacterial challenge resulted in a higher mortality. Li et al. (2005) reported a similar response to dopamine (except for the change in numbers of circulating hemocytes) in a different host (giant prawn) and parasite combination.

Catecholamines have been shown to affect the behavior of hemocytes from bivalves. Specifically, Lacoste et al. (2001) reported that 0.1 μM NE (and higher) inhibited phagocytosis in oysters, an effect that was mimicked by isoproterenol, but not by the alpha agonist, phenylephrine. The antagonist, propranolol (a beta-blocker), blocked the NE effect, but an alpha-blocker did not. Further experiments indicated that the reported effect was mediated via a cAMP-protein kinase A-dependent signal.

In the 1990s, Franchini and Ottaviani (1994) and Ottaviani et al. (1992d) published that ACTH induces cytoskeletal and motility changes of phagocytic hemocytes from snails. Genedani et al. (1994) basically confirmed these studies for CRF. They also reported that ACTH fragments (1–24), (1–4), (4–9), (1–13), (1–17), and (11–24) stimulate molluscan hemocyte migration, whereas the entire sequence (1–39) and the fragment (4–11) have an inhibitory effect. Differences between species were noted with respect to the response to individual fragments. Additionally, the (4–11) fragment could antagonize some of the stimulatory fragments (4–9) as well as TNF-α–induced chemotaxis. More recently, Malagoli et al. (2000) again confirmed (in the mollusc *Mytilus galloprovincialis*) that exogenous CRH provokes changes in the cellular shape of immunocytes and that this response is dependent on extracellular Ca^{++}. By using various inhibitors of transduction signaling pathways, they could completely or partially inhibit these changes. These findings are consistent with the proposition that PKA, PKC, and PKB/Akt are involved in CRH-induced cell shape changes in immunocytes and that the cellular effect of CRH needs the synergistic action of the two second messengers, cAMP and IP(3). In this study, Malagoli and colleagues also reported that immunocytes from the mussel express mRNAs for the CRH receptors, CRH-R1 and CRH-R2.

ACTH also causes molluscan hemocytes to release biogenic amines (NE, EPI, and dopamine) that influence chemotactic and phagocytic activities of hemocytes (Franchini and Ottaviani, 1994; Ottaviani et al., 1992d). The greatest release occurred after 15 minutes, but after 45 minutes, the values were similar to those of the controls. Culturing hemocytes with CRF also provoked release of biogenic amines, suggesting that endogenous ACTH mediates this release. These experiments also suggest that molluscan hemocytes have the capacity to bind and respond to CRH in a manner reminiscent of the way in which mammalian leukocytes respond to this releasing factor (Ottaviani et al., 1993a). These authors further demonstrated immunoreactive tyrosine hydroxylase and dopamine beta-hydroxylase (enzymes involved in biogenic amine biosynthesis) in these hemocytes. Ottaviani et al. (1994) found a similar but less significant catecholamine response when mammalian interleukin-2 (IL-2) rather than ACTH was added to cultures of hemocytes. Inter-

estingly, pre-incubation of hemocytes with IL-2 or with anti–IL-2 monoclonal antibody significantly reduced or completely eliminated the CRF-induced release of biogenic amines. Further direct evidence of competition between CRF and IL-2 was revealed by immunocytochemical and cytofluorimetric analysis. One explanation favored by these investigators (at least at that time) was the presence of a unique (ancestral?) receptor on molluscan hemocytes that is capable of binding both CRF and IL-2. If this is indeed the case, it would have significant implications for understanding the evolution of neural-immune system interactions. At the very least, these and other observations suggest that in terms of catechol biosynthesis, the invertebrate hemocyte may be a major player in an ancestral stress response that is associated with the HPA axis in mammals.

Stefano and colleagues (Dureus et al., 1993) also found that administration of mammalian neuropeptide Y (NPY) to either molluscan hemocytes or to human granulocytes inhibited both spontaneous activation and chemotaxis in response to the chemoattractant synthetic peptide, N-formyl-methionyl-leucyl-phenylalanine.

In a somewhat more recent investigation, Sassi et al. (1998) confirmed that ACTH (1–24) induces cell shape changes in the immunocytes of the mollusc, *Mytilus galloprovincialis.* Using computer-assisted microscopic image analysis, they reported that a G protein antagonist (suramin sodium), an adenylate cyclase inhibitor (2′,5′-dideoxyadenosine), and a protein kinase inhibitor (staurosporine) inhibited this effect. The highly specific inhibitors H-89 (for protein kinase A) and calphostin C (for protein kinase C) only partially inhibited the morphological alterations, whereas the simultaneous action of H-89 and calphostin C completely blocked them. Thus, mammalian ACTH-induced changes in cell shape appear to involve the adenylate cyclase/cAMP/protein kinase A pathway, as well as the activation of protein kinase C. In a related paper (Ottaviani et al., 1998b), ACTH receptor-like messenger RNA was detected in molluscan hemocytes (and, as a control, in human blood mononuclear cells) using a digoxigenin-labeled bovine cDNA probe. These findings imply that the ACTH receptor gene has been highly conserved during evolution and, according to these investigators, support their hypothesis that there is a phylogenetic relationship between the immune and neuroendocrine systems in invertebrates.

Stefano et al. (1989a) reported that opioids can also affect the behavior of hemocytes of the mussel, *Mytilus edulis.* Specifically, they found that the synthetic enkephalin analogue, DAMA (D-Ala^2, met^5-enkephalinamide), modulated locomotion, adherence, and conformation of a subset of hemocytes that resulted in their assuming a flattened and elongated conformation with extended pseudopodia. These morphological characteristics of hemocyte activation are similar to those seen following similar treatment of human granulocytes (Hughes et al., 1991b; Stefano et al., 1989a; Stefano et al., 1989b; Stefano et al., 1991a).

C. Effects of Mammalian Cytokines on Invertebrate Hemocytes

The third approach to studying invertebrate neural-innate immune system interactions has involved exploring the impact of molecules, purported to be homologues of mammalian pro-inflammatory cytokines, on hemocyte locomotion and phagocytosis, and on the production of nitric oxide synthase (NOS) and biogenic amines (Ottaviani et al., 1995c; Ottaviani et al., 1997). Some of these studies provide suggestive evidence that the cytokines tested can bind to, and compete with, CRF for the same membrane receptor (Ottaviani and Franchini 1995). However, given the known lack of cross-reactivity of most mammalian cytokines with cells from different mammalian species (Haynes and Cohen, 1991), these results with mammalian cytokines and invertebrate blood cells must remain more provocative than definitive.

D. Production of Cytokine-Like Molecules by Invertebrate Hemocytes

The final approach taken by these investigators addresses the production of cytokine-like molecules by invertebrate hemocytes in response to signals that clearly elicit cytokine production by mammalian leukocytes. Like human granulocytes, molluscan hemocytes respond to lipopolysaccharide (LPS) stimulation by assuming the active conformation changes described above (Hughes, et al., 1990; Hughes et al., 1991a; Hughes et al., 1991c). Similar LPS-induced changes of hemocytes from the insect *Leucophaea maderae* have also been published (Ottaviani et al., 1995e). At least for molluscs, this effect could be blocked by anti-mammalian TNF-α and/or anti–IL-1 antibodies. DAMA also is able to induce molluscan hemocytes to produce immunoreactive (ir)IL-1 (Stefano et al., 1991b). Administration of naloxone blocked the DAMA-induced conformational change by hemocytes, but these cells could still be activated by administration of recombinant human (rh)IL-1α, suggesting that opioid activation may be triggered by an IL-1–like molecule (Stefano et al., 1991b).

As mentioned earlier, molluscan hemocytes release biogenic amines when they are cultured with CRF, a

phenomenon that Ottaviani and co-workers (1991) described as a prototypic stress response. This response is significantly reduced when hemocytes are preincubated with IL-1α, IL-1β, TNF-α, or TNF-β prior to adding CRF to the incubation mixture (Ottaviani et al., 1995b).

Ottaviani and Franchini (1995) and Franchini et al. (1996a) used immunocytochemistry to detect immunoreactive platelet-derived growth factor α and β (PDGFα/β) and transforming growth factor (TGF)-β in phagocytic invertebrate leukocytes. The presence of PDGF-α/β–like receptors and TGF-β receptor (type II)-like molecules on the plasma membranes of the immunocytes of the mollusc *Mytilus galloprovincialis* was also suggested by immunocytochemistry (Kletsas et al., 1998). This latter study also revealed that PDGFα/β and TGFβ1 provoke changes in the shape of the molluscan hemocytes following interactions of these mammalian ligands with their putative receptors and that these extracellular signals are transduced along the phosphoinositide-signaling pathway. Ottaviani et al. (1998a) suggest that in the mussel, the major pathway followed by PDGFα/β and TGFβ in provoking the release of NE, EPI, and dopamine into cell-free hemolymph is mediated by a CRH-ACTH biogenic amine axis.

In mammals, microglial cells, like macrophages, are phagocytic and synthesize pro-inflammatory cytokines. Sonetti et al. (1997) argue that the snail, *Planorbius corneus,* also has a class of glial cells that resemble vertebrate microglia. Interestingly, these cells can be identified by their immunopositivity to anti–POMC-derived peptide antibodies. As in the vertebrates, snail microglia exhibit macrophage-like mobility, and when exposed *in vitro* to LPS or bacteria, they underwent conformational and mobility changes and also became phagocytic. Moreover, when activated, they also expressed TNF-α–like molecules and increased production of NOS, as shown immunocytochemically. Morphine (which appears to bind these cells via a μ3 receptor) inhibited this mobility and phagocytic activity of invertebrate microglia, suggesting to these investigators that opioid-like compounds may influence invertebrate microglia as well as hemocytes. Similar microglial-like cells have also been described in the mussel and the insect *Leucophaea maderae* (Sonetti et al., 1994). Excitability of a population of nociceptive sensory neurons in Aplysia were influenced by neighboring hemocytes (Clatworthy, 1998); Clatworthy and Grose (1999) suggest that *in vitro* activation of these hemocytes by LPS causes them to produce cytokine-like factors which modulate expression of injury-induced sensory nerve hyperexcitability.

The aforementioned studies with invertebrates are clearly provocative in terms of their suggesting a common evolutionary origin of the immune and neuroendocrine systems with their attendant inflammatory and stress responses (Ottaviani and Franchini, 1995; Ottaviani and Franceschi, 1996, 1997, 1998). However, before accepting the validity of this hypothesis, or even some of the data that led to its formulation, we must emphasize the importance of characterizing all the immunoreactive molecules and their receptors described in the previous paragraphs at the structural and genomic levels to determine if they are true homologues of their mammalian counterparts rather than, for example, an artifact of the detection methods used to identify them (Hahn et al., 1996).

A few intriguing studies we encountered in our review of the literature do not fit into the outline we've followed for this section on invertebrates. Indeed, they so beautifully reveal the evolutionary conservation of the links between behavior and its neuroimmune consequences that they merit their own paragraph. Mallon et al. (2003) and Riddell and Mallon (2006) presented behavioral evidence indicating a link between the immune system and the nervous system in insects. In brief, bumblebees that were injected with LPS to incite an anti-bacterial response in their hemolymph (Moret and Schmid-Hempel, 2000) have reduced abilities to learn (or recall memory) in a classical conditioning paradigm. Their study further points out that this associative learning deficit occurs only after bees are deprived of pollen (their only protein sources).

As will be discussed later in the section on birds, some evolutionary biologists have become interested in the immune system in general (and psychoneuroimmunology in particular) because of an interest in energy trade-offs between immune processes and various behaviors (e.g., reproduction, foraging for food, nest building, sexual displays). With respect to invertebrates, Fedorka et al. (2004) tested the hypothesis that "immune suppression" mediates a phenotypic trade-off between reproduction and immunity by manipulating reproductive effort and measuring immune function and mortality rates in the striped ground cricket, *Allonemobius socius.* In this species, male crickets provide females with a hemolymph-based "nuptial gift" during copulation. Based on their knowledge that hemolymph contains many immune mediators, these investigators predicted that sexual selection might differentially affect how disease resistance evolves in males and females. Indeed, they found that for both sexes, an increased mating effort resulted in a reduced immune ability. In their words, immune suppression appears to be a link between reproductive effort and cost in this system. Also of note are their observations that males and females differentially invest in several aspects of immunity prior to mating:

Males exhibit a higher concentration of circulating hemocytes and a superior bacterial defense capability than females. In a related study from this group, Zuk et al. (2004) reported that the ability of seasonal breeding crickets (both in the field and in the lab) to encapsulate foreign material was better in males than females. No sex dimorphism was noted in an aseasonal breeding species; however, when food was restricted, the males again did better. Although an interaction between the endocrine and defense systems are suspect in the results described in this and the preceding paragraph, no mechanisms have been explored formally. No doubt many experimental questions of potential psychoneuroimmunological interest raised by this intriguing line of research will keep these and other investigators gainfully occupied for several years.

III. NEURAL-IMMUNE INTERACTIONS IN TELEOST FISH

Given the phylogenetic success of the teleosts (Hickman et al., 1993), it would not be unreasonable to propose that fish, like all other ectotherms discussed in the following sections, display coordinated and integrated immune and neuroendocrine responses to environmental challenges. The information reviewed in this section and elsewhere (Weyts et al., 1999; Yada and Nakanishi, 2002) clearly support this hypothesis.

A. Innervation of Lymphoid Organs and Response of Leukocytes to Biogenic Amines

Autonomic innervation in the spleens of cod (Nilsson and Grove, 1974), coho salmon (Flory, 1989), and rainbow trout (Flory, 1990) has been demonstrated. At least for coho salmon, however, splenic innervation appears to be largely associated with the vasculature, with some branching out into the parenchyma (Flory, 1989). Chemical sympathectomy (SyX) of salmon with 6-hydroxydopamine (6-OHDA) depletes noradrenergic (NA) innervation as measured either by HPLC for NE, or by the absence of fluorescent NA nerve fibers (Flory, 1989) using sucrose-potassium phosphate-glyoxylic acid-induced (SPG) histofluorescence for catecholamines (de la Torre, 1980). Chemical SyX has also been reported to increase the number and percentage of splenic anti-sheep red blood cell (SRBC) plaque-forming cells (PFCs) in fish denervated prior to immunization; no effect was seen if immunization preceded SyX. These data are consistent with the augmented antibody response seen following SyX of neonatal rats (Besedovsky et al., 1979) and adult mice (Kruszewska et al., 1995, 1998), but contrast with the decreased antibody and cell-mediated responses seen after SyX in adult mice (Livnat et al., 1985; Madden et al., 1989a; Madden et al., 1989b; Sanders and Straub, 2002).

Flory (1990) also demonstrated for rainbow trout that adrenergic and cholinergic agents can alter *in vitro* antibody response to TNP-LPS. Specifically, the *in vitro* induction of a primary anti–TNP-LPS PFC response was suppressed by the β-adrenergic agonist, isoproterenol (10^{-4}–10^{-7} M), whereas it was enhanced by the α-adrenergic agonist, phenylephrine. The β-agonist effect could be blocked by propranolol, consistent with receptor mediation, and the α-agonist effect was blocked by yohimbine but not phentolamine. This suggestion of an α–2 adrenoreceptor was confirmed by the demonstration that clonidine (10^{-7}–10^{-11} M), an α–2 specific agonist, enhanced antibody responses. A cholinergic agonist also enhanced PFC responses over a dose range of 10^{-5}–10^{-11} M; this was blockable by the muscarinic antagonist, atropine. Subsequent studies have revealed an influence of adrenergic and cholinergic agents on the chemiluminescent and mitogenic responses of trout leukocytes (Bayne and Levy, 1991; Flory and Bayne, 1991). Plytycz and co-workers (Józefowski and Plytycz, 1998; Józefowski et al., 1995) extended Flory's studies by demonstrating first that there are adrenergic and cholinergic receptors on head kidney leukocytes of the goldfish, *Carassius auratus;* and second, that high concentrations of the β-adrenergic agonist, isoproteronol (10^{-4} M), and the cholinergic agonist, carbachol (10^{-5} M), enhanced phorbol myristate acetate (PMA)-induced oxidative burst of goldfish macrophages, effects that could be blocked by equimolar concentrations of propranolol and atropine, respectively. Both EPI and NE enhance the respiratory burst activity of carp anterior kidney macrophages and neutrophils (Verburg-van Kemenade, personal communication). Finally, Narnaware and colleagues (Narnaware and Baker, 1996; Narnaware et al., 1994) observed that both α- and β-adrenergic agonists depress *in vitro* phagocytosis of yeast by rainbow trout macrophages and that injection of the adrenergic blocker phentolamine can prevent the depressive effects of "stress" on the phagocytic index of cells from the same species.

Serotonin (5-HT) is also immunomodulatory in fish. According to a set of detailed experiments (Ferriere et al., 1996), 5-HT suppressed LPS- and PHA-induced proliferation of trout PBLs. This inhibitory effect could be mimicked by an agonist of 5-HT1A receptors (8-OH-DPAT) and was reversed by an antagonist of 5-HT1A and 5-HT1B receptors (spiperone). Scatchard plot analyses confirmed the existence of specific sero-

tonin receptors on lymphocytes. In a competition study, serotonin inhibited the binding of ^{3}H-5HT to receptors in both resting and mitogen-stimulated lymphocytes. However, the agonists (8-OH-DPAT and buspirone) and antagonist (NAN-190) of the 5-HT1A receptor subtype failed to displace ^{3}H-5HT binding to receptor sites in resting cells, but they did inhibit ^{3}H-5HT binding in LPS- and PHA-stimulated lymphocytes. Based on these observations, the authors propose that 5-HT1A receptors are expressed on activated lymphocytes only after mitogenic stimulation. An agonist of 5-HT1B receptors (CGS-12066B) failed to affect ^{3}H-5HT binding on either resting or mitogen-stimulated lymphocytes, suggesting that this 5-HT receptor subtype is absent on lymphocytes. A subsequent pharmacological study from the same group (Meyniel et al., 1997), in which additional antagonists of mammalian 5-HT receptors (ICS-205–930 and metoclopramide) were used, suggests that fish 5-HT3 lymphocyte receptors may differ pharmacologically from mammalian receptors.

B. Neuropeptide Production by Cells of the Teleost Immune System

Recently, investigators have begun to explore whether fish leukocytes, like mammalian lymphocytes (Blalock, 2005), synthesize hormones typically associated with the hypothalamo-pituitary-interrenal (HPI) gland axis.[1] POMC-derived peptides (ACTH, α-MSH, and β-endorphin) have been detected immunocytochemically in goldfish thymic epithelial cells (Ottaviani et al., 1995d), and constitutive and mitogen-stimulated production of immunoreactive POMC products by catfish lymphocytes has also been reported (Arnold and Rice, 1997). Since both studies were based on antibody detection of antigenic cross-reactivities, their interpretation may be questioned. However, Ottaviani et al. (1995a) have also reported that goldfish (*C. auratus*) phagocytic leukocytes express POMC mRNA as determined by *in situ* hybridization with a digoxigenin-labeled human DNA probe. The same probe also detected POMC mRNA in phagocytic leukocytes and peripheral blood lymphocytes from the frog, *Rana esculenta,* but lymphocytes from goldfish did not express this gene. A study of different teleost and amphibian species, however, seems necessary before endorsing these authors' suggestion that expression of this gene in vertebrate lymphocytes first occurred in the Amphibia.

A major contribution to psychoneuroimmunology in the past decade has been research revealing that in mammals communication between the neuroendocrine and immune systems is mediated, at least in part, by pro-inflammatory cytokines (e.g., IL-1, IL-6, TNF-α). For example, in rodents, IL-1 can act on the hypothalamus and pituitary to elicit CRH and ACTH, respectively (Parsadaniantz et al., 1994, 1997). Thanks largely to the work of Secombes and colleagues in Scotland, several cytokine genes that include IL-1 (Pleguezuelos et al., 2000; Secombes et al., 1998); IL-8 (Laing et al., 2002); IL-10 (Zou et al., 2003); TNF-α (Zou et al., 2002, 2003); IFN-γ (Zou et al., 2005); IL-6 (Bird et al., 2005); lymphotoxin b (Kono et al., 2006), IL-11 (Wang et al., 2005); and IL-18 (Zou 2004) have been cloned in different species (e.g., salmon, rainbow trout) and, at least for some genes, their products expressed as recombinant proteins (reviewed in Secombes et al., 2001; Secombes and Cunningham, 2004). This major sustained effort has permitted research on the role of cytokines as mediators of neuroendocrine-immune interactions in teleosts. Indeed, there is now solid evidence that in fish, IL-1β is an effector molecule that activates the HPI axis as evidenced by a strikingly elevated level of plasma cortisol in trout receiving an i.p. injection of 0.1–0.6 nmol/kg of the recombinant protein (Engelsma et al., 2002; Holland et al., 2002). Holland et al. (2002) also reported that trout IL-1β peptides (P1 and P3), which are homologous to receptor-binding sequences of human IL-1β, failed to influence the prevailing cortisol concentration even though an equivalent dose was immunostimulatory *in vivo.* In this study, blockade of endogenous ACTH release by administration of the synthetic glucocorticoid dexamethasone (DEX) prevented the rIL-1β–mediated elevation of plasma cortisol. Inhibition studies with the cloned fish IL-1 receptor-associated protein and IL-1 receptor (Stansberg et al., 2005) have yet to be carried out to further probe the role of this cytokine in the neuroimmune circuitry. Nevertheless, the important phylogenetic take-home message from these studies is that IL-1 signaling between the immune and neuroendocrine systems in mammals is conserved in lower vertebrates. Just how early this neuroimmune regulatory pathway evolved (i.e., is it present in elasmobranchs and agthanans?) remains unresolved. So too does the question of whether, like mammals, other pro-inflammatory cytokines play a signaling role at the teleost level of phylogeny.

LPS is known to activate the fish HPI axis (Balm, 1997). Since increased cortisol levels could be induced by either an i.p. injection of LPS or rIL-1β, it is reason-

[1]Since in fish, the interrenal glands serve the function provided by the adrenal cortex in mammals (Chester Jones et al., 1980), the hypothalamo-pituitary-adrenal axis and the hypothalamo-pituitary-interrenal axis are functionally equivalent.

able, by extrapolating from the mammalian literature, to conclude that in fish LPS induces an increase in IL-1 that, in turn, effects increased levels of cortisol via activation of the HPI axis. An early literature points out that direct exposure of pituitary tissue to LPS *in vitro* blunts ACTH and α-MSH release (Balm et al., 1995). Although it is once again reasonable to assume that these changes result from an increase in cytokine secretion following LPS treatment, it should be noted that LPS can also affect endocrine tissues directly (Brunetti et al., 1994; Milton et al., 1993).

One of the most important reasons for conducting studies using a homologous system (i.e., trout rIL-1b injected into trout) is that it obviates problems inherent in using mammalian cytokines in fish. Earlier studies like one in which murine IL-1α was reported to inhibit α-MSH release by the HPI axis (Balm et al., 1993) or studies pointing out that mammalian IL-1 has no effect on teleost lymphocytes (see review by Haynes and Cohen 1991; see also Ellsaesser and Clem, 1994; Verburg-van Kemenade et al., 1995) need to be repeated in the homologous system, especially since mammalian and fish IL-1 have minimal identity at the DNA and amino acid sequence levels (Secombes et al., 1998).

C. Glucocorticoids, Neuropeptides, and Stressor Effects on Immunity

The neuroendocrine stress response of fish (Wendelaar Bonga, 1997) is quite similar to that of mammals (Chrousos and Gold, 1992) in that it consists, in part, of a stressor-sensitive HPI axis. Cortisol, the major glucocorticoid in fish, is produced by the interrenal gland. Primary mediators of cortisol in teleosts are ACTH (apparently for acute stress situations) and α-MSH (in more chronic situations) (Donaldson, 1981; Lamers et al., 1994; Sumpter et al., 1994). These hormones, in turn, are under hypothalamic control via CRF for ACTH (Olivereau and Olivereau, 1991) or via both CRF and TRH α-MSH (Lamers et al., 1994). Parenthetically, in an *in vitro* study, Harris and Bird (1998) demonstrated that a 1-hour exposure to α-MSH increases the phagocytic ability of head kidney macrophages and neutrophils from rainbow trout.

Although the neuroendocrinology of the stress response in teleosts has been well studied (Huising et al., 2004, 2005; Rotlant et al., 2003; van den Burgh et al., 2005), information that causally relates, in a stepwise and mechanistic fashion, stressor-associated neuroendocrine levels and pathways to changes in immune system parameters and susceptibility to pathogens is relatively fragmentary. This is not to say that there is a dearth of studies that explore the effects of stressors on selected aspects of immune function and on mortality in agriculturally important teleost species. Quite the contrary (Barton and Iwama, 1991; Ellis, 1981; Ndoye et al., 1991; Pickering, 1981; Wendelaar Bonga, 1997). For example, a 30-second dip net removal of Chinook salmon from the water temporarily elevated plasma cortisol, increased leukocyte numbers in the thymus and anterior kidney, decreased blood and spleen leukocytes, and altered resistance to the fish pathogen *Vibrio anguillarum* (Maule et al., 1989). Altered resistance was manifested by an increased mortality and decreased time-to-death in salmon exposed to *Vibrio* 4 hours after stressor exposure and by a decreased mortality (relative to controls) and longer survival times in fish exposed 1 day after this acute stressor (Maule and Schreck, 1990a; Maule et al., 1989). Altered immune function was also reflected by a decreased *in vitro* anti-TNP antibody production (relative to unstressed fish) by anterior kidney leukocytes 4 hours and 7 days after stressor exposure. At this later time-point, plasma cortisol levels had returned to normal (Maule et al., 1989), indicating that the effects of the stressor persist beyond the time of cortisol elevation. A similar observation was made by Betoulle et al. (1995), who subjected trout to hyperosmotic shock for 7 days or 30 days and measured cortisol, PRL, and anti-*Yersinia ruckeri* antibodies in the serum. Relatively short-term "stress" was associated with a correlation between high levels of both stress hormones and a delayed production and lower titers of antibody. More chronically exposed animals had no increase in stress hormones but still had low antibody titers. These two reports, among others, are consistent with the idea that the putative immunomodulatory effects of stress hormones impact the earlier phases of antibody production.

A recent study (Binuramesh et al., 2006) has convincingly demonstrated that the social environment of fish plays an important role in their adaptive and innate immune responses to pathogens. This research examined the effects of sex ratio of a tilapia (*Oreochromis mossambicus*) housed for 4 weeks either as same sex or mixed (1:1) sex cohorts on antibody responses to *Aeromonas hydrophila;* serum lysozyme activity; production of intracellular reactive oxygen species (ROS); on reactive nitrogen species (RNS) by peripheral blood leukocytes; and on disease resistance against live, virulent *Aeromonas hydrophila.* Their data convincingly showed an enhanced antibody response and increased number of antibody-producing cells in the mixed-sex cohorts relative to fish in monosex ratio groups. Similar enhancement was also observed in non-specific immune parameters such as serum lysozyme level, ROS, and RNS production. The host resistance test

revealed that enhanced immunity in the equal male and female sex ratio group was protective against *Aeromonas hydrophila* infection. Hence, natural sex ratios may enhance disease resistance to pathogens in this species; just how it does so is an important but unresolved issue. As indicated in the aforementioned Binuramesh study, stressors can modify various aspects of the innate as well as adaptive immune system of teleosts. In another example from other species (e.g., trout), *in vitro* respiratory burst activity of fish anterior kidney phagocytes was diminished following handling and exposure to anoxic shock, as well as by crowding (Angelidis et al., 1987; Pulsford et al., 1994; Yin et al., 1995), but it was increased in trout following transfer from fresh to sea water (Marc et al., 1995), indicating a variance across species and/or stress modalities. A social stress paradigm in rainbow trout led to increased *in vivo* phagocytosis of bacteria by peripheral blood phagocytes (Peters et al., 1991). Transition from fresh to seawater, however, had no effect on activity of natural cytotoxic cells (NCC) isolated from brown trout (Marc et al., 1995). Although decreasing the water temperature in which carp were held enhanced their NCC activity (Le Morvan-Rocher et al., 1995), the *in vitro* assays were all performed at 28°C, which could have different consequences for cells isolated from fish adapted to different temperatures (Clem et al., 1984, 1991). A social stress paradigm in aggressive fish (*Tilapia*) resulted in depressed NCC activity and mitogenic responses in the subordinate fish (Ghoneum et al., 1988). As determined by blocking studies with naltrexone, this effect seems to be mediated, at least in part, by endogenous opioids (Faisal et al., 1989). A group of Polish investigators led by Plytycz has demonstrated that endogenous opioids (i.e., morphine) also appear to be involved in reducing numbers, but increasing respiratory burst activity, of thioglycollate-elicited inflammatory cells in peritoneal exudates from goldfish (Chadzinska et al., 1997; Gruca et al., 1996) and salmon (Chadzinska et al., 1999). This morphine-induced increase in respiratory burst activity did not occur if the exudate cells were harvested from stressed salmon (Plytycz et al., 1996). As would be predicted by the results from these studies involving the parenteral administration of morphine and naltrexone, opioid receptors have been identified on teleost head kidney leukocytes cells and characterized by radiolabeled ligand binding (Józefowski and Plytycz, 1997).

As in mammals, lysozyme plays a non-specific antibacterial defense role in fish. Plasma lysozyme levels (as well as plasma cortisol and epinephrine) in rainbow trout increased following 30 seconds of handling (Demers and Bayne, 1997). Lysozyme levels were also increased in brown trout following their transition from fresh to seawater (Marc et al., 1995) but were unaffected by parr-smolt transformation (see below) in Atlantic salmon (Olsen et al., 1993) and were actually decreased in carp following 30 days of crowding (Yin et al., 1995). These differences may well relate to the duration and severity of the stressor, as was shown by Möck and Peters (1990), who observed that 30 minutes of handling increased lysozyme levels in rainbow trout, whereas a 2-hour transport stressor decreased their levels.

It seems clear that many of the effects of stressors on non-specific and specific defense modalities in fish involve the HPI axis and glucocorticoids. Fish leukocytes possess receptors for corticosteroids (Maule and Schreck, 1990a, 1990b; Verburg-van Kemenade et al., 1999; Weyts et al., 1998a). In coho salmon, receptor-like binding of a synthetic corticosteroid analogue (triamcinolone acetonide) to cells isolated from spleen and head kidney was reported (Maule and Schreck, 1990b). Carp peripheral blood cells also express cortisol receptors with a high binding affinity (Kd 3.8 nM). Neutrophilic granulocytes isolated from the carp head kidney contain cortisol-binding sites with the same characteristics (Weyts et al., 1998a, 1998c), suggesting that both PBL and head kidney neutrophils express the same glucocorticoid receptor. Basal receptor densities in both cell types are approximately 500 per cell. Following cortisol treatment *in vivo,* receptor numbers in carp PBL decrease (Weyts et al., 1997), whereas numbers of corticosteroid receptors in coho salmon spleen and head kidney leukocytes increase following exposure to an acute or chronic stressor or by cortisol treatment *in vivo* (Maule and Schreck, 1991). These changes in receptor densities have been explained by a stress- or cortisol-induced trafficking of receptor-rich leukocyte subtypes from the circulation into lymphoid organs. However, since corticosteroid receptors in coho salmon head kidney leukocytes are also increased following an *in vitro* exposure to cortisol (Maule and Schreck, 1991), an actual upregulation of receptor numbers resulting from the cortisol treatment also seems reasonable.

The immunosuppressive effects of glucocorticoids have been demonstrated in several studies with teleosts. Anderson et al. (1982) injected rainbow trout with a synthetic glucocorticoid 24 hours after immunizing them with the O-antigen of *Yersinia ruckeri* and observed depressed *in vitro* and *in vivo* antibody production and a reduced number of splenic lymphocytes. A similar reduction in numbers of antibody-producing cells has been described in flounder (Carlson et al., 1993). Ellsaesser and Clem (1987) injected channel catfish i.v. with cortisol (6.7 µg/kg body weight),

which resulted in a plasma level of cortisol 30 minutes following injection equivalent to that seen 30 minutes following "transport stress" in this species. This increase correlated with decreased numbers of circulating leukocytes, increased neutrophils, and decreased LPS- and Con A-induced lympho-proliferation. This last observation has also been made for salmon by Espelid et al. (1996). Since these Norwegian investigators noted that the addition of physiologic levels of cortisol to normal fish leukocytes *in vitro* did not alter mitogen responses, they suggested that an indirect mechanism was involved in the observed effects. However, Tripp and colleagues (1987) found that physiological concentrations of cortisol *in vitro* did, in fact, depress both LPS-induced mitogenesis and the primary anti–TNP-LPS antibody responses of splenic and head kidney lymphocytes from coho salmon. In this paradigm, pronephric lymphocytes were sensitive early in the antibody response, whereas splenic lymphocytes were sensitive throughout the culture period. Although it is unknown whether the aforementioned differences in the *in vitro* effects of cortisol on salmon and catfish lymphocyte mitogenesis are related to the species used or to methodological considerations, others have also shown that *vitro,* cortisol inhibits teleost lymphocyte proliferation (Grimm, 1985; Pulsford et al., 1995; Tripp et al., 1987) and reduces antibody production (Tripp et al., 1987; Wechsler et al., 1986).

It has been suggested that cortisol may act on fish lymphocytes by inhibiting cytokine production as it does in mammalian cells (Kaattari and Tripp, 1987; Tripp et al., 1987). On the other hand, the observation that *in vivo* cortisol treatment resulting in plasma concentrations of 400 ng/ml enhances the numbers of apoptotic lymphocytes in the skin of rainbow trout (Iger et al., 1995) suggests that apoptosis may be regulated by cortisol. Indeed, apoptosis appears to have been conserved as an immune regulatory mechanism in fish as well as other ectothermic vertebrates including frogs (Haberfeld et al., 1999; Rollins-Smith, 1998; Rollins-Smith and Blair, 1993; Rollins-Smith et al., 1997a; Ruben et al., 1994) and salamanders (Ducoroy et al., 1999). Cortisol-induced apoptosis of fish leukocytes is mediated by a glucocorticosteroid receptor since the glucocorticoid receptor antagonist RU486 (Weyts et al., 1998a, 1998b, 1998c) could block apoptosis. The low concentration of cortisol (0.1 μM) that was effective in inducing B-cell apoptosis contrasts with the lack of effects of cortisol's natural conversion product, cortisone. Since the conversion of cortisol to cortisone in fish is highly preferred over the reverse reaction (Donaldson and Fagerlund, 1972), this conversion may provide the fish with a mechanism to regulate the effects of corticosteroids on cells of the immune system. The lack of apoptosis induction by cortisone correlates with the low affinity of the glucocorticosteroid receptor in carp PBL for cortisone (250 times lower than that for cortisol) (Weyts et al., 1998c).

Effects of cortisol on leukocyte viability are cell type-specific. For example, B-cells from carp are especially sensitive to cortisol, whereas thrombocytes and T-cells are insensitive. Induction of apoptosis depends on the developmental state and/or activation state of the lymphocyte. In the periphery, only activated B-cells appear sensitive, whereas in head kidney and spleen apoptosis induction in B-cells is independent of the activation state (Verburg-van Kemenade et al., 1999).

Interestingly, *in vitro* apoptosis of carp head kidney neutrophils was reduced when cells were cultured with cortisol, and this effect of cortisol was also mediated by a glucocorticoid receptor (Weyts et al., 1998b). Analysis of the glucocorticoid receptors in these cells revealed that they may be the same as those detected in PBLs since both have the same affinity and specificity (Weyts et al., 1998c). The inhibition of neutrophil apoptosis by cortisol, combined with the observation that neutrophil respiratory burst activity was not affected by cortisol, would augment the supply of functional neutrophils in stressful conditions. Taking into account that neutrophils, together with macrophages, form the first line of defense against invading microorganisms (Dalmo et al., 1997), mobilization of these cells under stressful conditions may be important for survival.

Although cortisol can trigger apoptosis of leukocytes, and stressors elevate this steroid in fish, Alford et al. (1994) observed that confinement stress of channel catfish was associated with a decrease in apoptotic PBLs, and *in vitro* culture of lymphoid cells with cortisol failed to induce apoptosis. This apparent discrepancy with some of the previously cited literature may relate to the fact these investigators used unstimulated cells in their experiments and, at least in carp, only mitogen-stimulated PBLs are sensitive to cortisol-induced apoptosis (Weyts et al., 1998b).

Plasma cortisol concentrations in stressed salmonids and cyprinids range between 100 and 500 ng/ml (Barton and Iwama, 1991), of which approximately 25–125 ng/ml is present in an unbound configuration (Caldwell et al., 1991; Flik and Perry, 1989). Therefore, concentrations in the micromolar range or higher may not be physiological. It appears that *in vitro,* cortisol does not affect phagocytosis or respiratory burst activity (Narnaware et al., 1994; Weyts et al., 1998b) unless

supraphysiological concentrations in the micromolar range (or higher) are used (Ainsworth et al., 1991; Pulsford et al., 1995; Stave and Roberson, 1985). Accordingly, respiratory burst activity of a goldfish macrophage cell line was unaffected by up to 10 μM cortisol (Wang and Belosevic, 1995). The inhibition of phagocytosis of SRBC that was described in the same study was again only detected at relatively high (1 μM) cortisol concentrations.

Studies of cellular immune functions associated with either stressor administration or *in vivo* cortisol treatment often fail to consider leukocyte trafficking and redistribution as an explanation of the apparent immunosuppression observed *in vitro.* A wealth of information reveals that many stressors (e.g., transport, anoxia, social conflict, handling, injection) in several fish species are associated with decreased numbers of circulating B-lymphocytes and increased numbers of circulating neutrophils (Ainsworth et al., 1991; Angelidis et al., 1987; Bly et al., 1990; Ellsaesser and Clem, 1987; Espelid et al., 1996; Faisal et al., 1989; Pulsford et al., 1994; Salonius and Iwama, 1993). These effects are mimicked by *in vivo* corticosteroid treatment (Ainsworth et al., 1991; Ellsaesser and Clem, 1987; Espelid et al., 1996; Weyts et al., 1997). Increased infiltration of leukocytes into the thymus, head kidney, skin, and gill (Balm and Pottinger, 1993; Iger et al., 1995; Maule and Schreck, 1990a; Peters et al., 1991) has also been observed following either stress or *in vivo* cortisol administration. Thus, interpretation of data obtained from *in vitro* functional analysis of leukocytes from stressed fish or from fish injected with cortisol needs to take into consideration the possible (dis)appearance of cell populations rather than simply changes in cell activity.

Although there are more data supporting a neuroendocrine-immune link in fish than there are for any other non-mammalian vertebrate, studies to date have used many different species and many different types of stressors. Thus, no "best use" model has emerged to allow an in-depth study of the effects of various sorts of stressors on several immune parameters of a single species. Effects are leukocyte type dependent, and the final outcome may depend on the severity and duration of the stressor, as it does in mammals (Moynihan et al., 1994). The cortisol-mediated rescue of neutrophils from apoptosis shows that cortisol does not suppress all aspects of the fish defense system. Rather, cortisol acts as a regulator, inhibiting some parts of the (specific) immune response and enhancing other (nonspecific) components that may be functional in stressful situations. Stimulation of an innate immune response may be part of an adaptive response necessary to combat potential pathogens under stressful conditions (Weyts et al., 1999).

D. Seasonal Influences on Immunity

Smolting, a series of profound physiological changes that prepare juvenile freshwater salmon for entry into salt water, is characterized by increases in plasma thyroxine and cortisol levels (Maule et al., 1987). These hormonal changes correlate with decreased numbers of splenic PFCs in salmon immunized with the O-antigen from *V. anguillarum* and also with decreased numbers of PBL (although there was an increase in the proportion of small lymphocytes) relative to either erythrocytes or fish body weight. Such changes, together with increased mortality to *Vibrio* infection, have also been seen following implantation of cortisol-containing pellets (Maule et al., 1987).

Like reptiles and amphibians (discussed in following sections), immune reactivities and lymphoid tissues of teleosts undergo seasonal changes that are unrelated to smolting. For example, Yamaguchi et al. (1981) found that the agglutinating and cytotoxic antibody responses of trout immunized in the spring with the pathogen *Aeromonas salmonicida* were higher and increased more rapidly than those of fish immunized in the winter, even though animals were held at a constant temperature of 18°C. Seasonal modulation in antibody production in relation to the state of lymphoid tissue development has also been studied in the ovoviviparous marine fish, *Sebastiscus marmoratus* (Nakanishi, 1986). Fish immunized with SRBC in summer after having been acclimated for at least 2 weeks to 23°C, had higher antibody titers than fish immunized in winter, even when the environmental temperature of acclimation and immunization was constant. A sexual dimorphism was noted in that anti-SRBC antibody titers of mature females were lower than that of either males or immature females in the winter spawning season. In addition, the thymus of pregnant and especially post-spawning females was entirely involuted, showing a marked decrease in the number of lymphocytes in both the cortex and medulla. The neuroendocrine regulation of such dramatic changes seems well worth further study.

Circadian rhythm has been shown to influence immune responses in fish. The gulf killifish, *Fundulus grandis,* for example, exhibits a circadian variation in immune reactivity during scale allograft rejection. Specifically, a two- to three-fold higher level of immune activity and cellular destruction occurred during the dark period, resulting in a longer survival time for grafts transplanted at light onset than for those grafted

at lights off (Nevid and Meier, 1993, 1994). Phase relationships between two circadian neuroendocrine oscillations (daily photoperiod and non-photic daily stimuli) appear to be involved (Nevid and Meier, 1995a), as do levels of hormones and neuropeptides/neurotransmitters (Nevid and Meier, 1995b). For example, daily rhythms of alloimmune reactivity could be abrogated by treating fish with naloxone or propranolol at light offset only, GH or atropine at light onset only, or PRL at either light onset or light offset. Timed treatments with PRL or GH reduced the length of time needed to completely destroy scale grafts, whereas timed treatments with propranolol or naloxone prolonged graft survival (Nevid and Meier, 1995b).

IV. NEURAL-IMMUNE INTERACTIONS IN AMPHIBIANS

Based on a variety of morphological and physiological characteristics that distinguish Amphibia from Teleosteii and Reptilia, this class of vertebrates is generally thought of as a phylogenetically pivotal group. As such, the immune systems of a few (one hopes representative) amphibian species have been exhaustively studied. In the last decade, a few "amphibian immunologists" have broadened their research focus to include neural-immune interactions in both frogs and salamanders. Areas being investigated include: (a) innervation of lymphoid tissues; (b) neuropeptide/neurotransmitter regulation of immunity; (c) seasonal/neuroendocrine effects on immunity in adults; (d) neuroendocrine regulation of immunity during metamorphosis; and (e) the impact of environmental stressors on anti-microbial immunity and its implications for the worldwide decline of amphibians.

A. Innervation of Lymphoid Organs

Several lines of evidence point to catecholamines as "neuroimmune transmitters" in amphibians. Prior to the published observations in mammals by the Feltens in the 1980s (Felten and Olschowka, 1987; Felten et al., 1987), Nilsson (1978) had used fluorescence histochemistry to reveal sympathetic innervation of the spleen of the cane toad, *Bufo marinus,* and Zapata et al. (1982) published electron microscopic evidence of direct contacts between nerve endings and lymphoid cells in the jugular body of the leopard frog, *Rana pipiens* (Manning and Horton, 1982). More recently, Kinney et al. (1994b) described noradrenergic and peptidergic innervation of the spleen of the adult South African clawed frog, *Xenopus laevis,* using: SPG histofluorescence (de la Torre, 1980) for catecholamines and immunocytochemistry for tyrosine hydroxylase (TH), the rate-limiting enzyme in the synthesis of catecholamines (Felten and Olschowka, 1987); PGP 9.5 (a general neuronal antigen); and NPY. Spleens of this species, like those of other anurans, have a clearly defined red and white pulp (Manning, 1991; Manning and Horton, 1982). Noradrenergic fibers are almost exclusively restricted to the white pulp in association with the central artery. These fibers have occasional varicosities in the parenchyma, and additional fibers are present in the boundary cell/perifollicular areas where they may come into contact with B-cells and macrophages of the white pulp; non-lymphoid dendritic cells involved in trapping and retention of soluble antigen (Manning, 1991); and possibly the T-cells at the extreme boundary of the white pulp. In some instances, fibers were also noted in the splenic capsule. In short, this innervation pattern in *Xenopus* is similar to that described for the murine spleen (Felten et al., 1987).

The profile of fine varicose nerve fibers staining for NPY in the white pulp of the *Xenopus* spleen was similar to, but less abundant than, TH^+ fibers, an observation that may reflect a difference in sensitivity of the antibody used rather than an actual difference in amount of neurotransmitter present. Substance P-staining fibers were also found around vessels in the splenic white pulp (Kinney et al., 1994b).

Although frogs, toads (Anura), and salamanders (Urodela) are all amphibians, the two taxonomic orders differ immunologically in several interesting ways, such as the delayed kinetics of antibody production and allograft rejection characteristic of urodeles relative to anurans (Cohen and Koniski, 1994). In addition, and in sharp contrast to the mammalian-type pattern of innervation that characterizes the compartmentalized *Xenopus* spleen, SPG histofluorescence analysis of the non-compartmentalized spleens of the salamanders *Taricha torosa, Notophthalmus viridescens,* and *Ambystoma mexicanum* revealed a diffuse pattern of innervation associated with the reticular network (Kinney et al., 1994b).

The third order of amphibians, the Gymniophiona (Apoda or caecelians), has received only limited attention from immunologists, no doubt because they are difficult to obtain for laboratory research. The spleen of one such apodan, *Typhlonectes sp.*, is elongate like that of salamanders. Unlike salamander spleens, however, the *Typhlonectes* spleen exhibits some aggregation of lymphocytes into white pulp-like regions that are less organized than the white pulps of the anuran or mammalian spleen (Manning, 1991). The spleen of this species is also characterized by less abundant innervation than the urodele spleen, both in

immediate proximity to blood vessels and also in areas removed from blood vessels, as shown by SPG histofluorescence. PGP 9.5 staining of the *Typhlonectes* spleen revealed occasional individual fibers and fiber bundles in a pattern similar to that seen with histofluorescence (Kinney et al., 1994b).

The ontogeny of splenic innervation during the larval life of *Xenopus* has also received some attention. Clothier et al. (1991) reported changes in splenic innervation during the period shortly before metamorphic climax. Specifically, they described a drop in the levels of splenic NE, as assessed by HPLC and SPG histofluorescence at Nieuwkoop and Faber (1967) larval stage 58, a time when lymphocyte mitogen responsiveness is also significantly decreased (Rollins-Smith et al., 1984). Unfortunately, this reported developmental loss of splenic NE was not accompanied by micrographic documentation of a loss of sympathetic nerve fibers in the spleen. The lack of such documentation becomes important in view of our observations (Kinney, 1996) that the larval *Xenopus* spleen is innervated earlier than stage 58 (i.e., from stage 54 onward), and that the appearance of innervation is very sensitive to the environmental conditions (e.g., temperature, animal density) under which the larvae are reared. We have also reported that chemical SyX during larval life prior to the appearance of splenic compartmentation does not affect the subsequent development of the demarcation into a red and white pulp (Kinney, 1996), and that early larval thymectomy that renders animals T-cell deficient does not influence the normal development of innervation (Kinney et al., 1993; Rollins-Smith and Cohen, 1995).

B. Sympathetic and Neuroendocrine Regulation of Immunity

SyX (using 6-OHDA) of adult *Xenopus* is associated with a significant increase in the *in vitro* proliferative response by splenocytes cultured with the mitogens LPS, Con A, and PMA (Kinney, 1995; Kinney and Cohen, 2005). A similar increase in *in vitro* proliferation was noted when splenocytes from frogs that had been parenterally immunized with keyhole limpet hemocyanin (KLH) 2 days after SyX were cultured with KLH (Kinney, 1995). This SyX-associated enhancement of polyclonal- and antigen-driven proliferation of *Xenopus* splenocytes is similar to observations in mice (Kruszewska et al., 1995, 1998). Unlike these murine studies, our initial (Kinney, 1995) SyX experiments did not reveal any alteration in the primary serum anti-KLH IgM antibody response (assayed 1–55 days post-immunization) in frogs immunized 2 days after 6-OHDA treatment. This apparent lack of effect was recently reproduced (Kinney, unpublished). However, when such primed frogs were again treated with 6-OHDA 47 days after the initial SyX and injected with a second dose of KLH 2 days later, the secondary IgY anti-KLH response was increased relative to the appropriate controls. To the best of our knowledge, the impact of SyX on secondary antibody responses in other species has never been examined.

SyX does not appear to affect the time-course of skin allograft rejection (Kinney, 1995; Kinney et al., 1994a), a T-dependent immune process in *Xenopus* (Manning et al., 1976). Specifically, injection of 6-OHDA 2 days before transplantation, and repeated weekly during the course of the experiment, did not affect skin graft survival, regardless of whether donor and hosts differed by MHC plus minor histocompatibility (H) locus antigens or by minor H-antigens only. There was also no effect of SyX on the accelerated second-set rejection of minor H locus-disparate grafts. In confirmation of these data, Józefowski et al. (1996) reported that chronic *in vivo* administration of β-adrenergic (propranolol) or muscarinic (atropine) antagonists had no effect on skin allograft survival in *R. esculenta* and *R. temporaria.* Morphine, too, was without effect. The picture is slightly different, however, when the fate of xenografts rather than allografts was investigated using *R. esculenta* as hosts. Specifically, repeated injections of propranolol increased the survival time of xenogeneic skin grafts from *R. temporaria* and *B. bomina,* injections of atropine significantly accelerated rejection of skin from *B. bomina* but not *R. temporaria,* and injections of morphine had no effects regardless of the donor species used. Interestingly, binding of radiolabeled ligands to muscarinic and adrenergic receptors on PBLs was increased significantly in xenografted animals but not on cells from recipients of allografts. It is also noteworthy that unlike classic T-cell–mediated rejection of allografts, xenograft rejection in anurans is thought to primarily involve innate and antibody-mediated immunity (Horton et al., 1992; Józkowicz., 1995).

In *Xenopus,* immunological tolerance characterizes the alloimmune response of perimetamorphic animals to skin grafts from adult donors that differ from the hosts only by minor H-antigens (DiMarzo and Cohen, 1982). SyX of newly metamorphosed recipients of such grafts had no effect on either the induction or maintenance of this non-deletional form of tolerance (Kinney, 1995).

As in so-called higher vertebrates, immunological effects of SyX suggest that cells involved in amphibian immunity express receptors for noradrenergic ligands. Indeed, in the late 1970s, an English group (Hodgson et al., 1978, 1979) reported that antigen-binding splenocytes from several species of SRBC-immunized sala-

manders (*T. cristatus, T. alpestris, Cynops hongkongensis, C. pyrrhogaster, N. viridescens*) were decreased following their *in vitro* exposure to both α- and β-adrenergic receptor (AR) stimulation[2]. In immunized frogs (*R. temporaria, X. laevis, R. esculenta, Bufo bufo*), α-AR stimulation decreased, whereas β-AR stimulation increased the number of antigen-binding splenocytes. In these early studies, it was erroneously assumed that all antigen-binding cells were antibody-producing cells. Subsequently, this group (Clothier et al., 1989, 1992) used an ELISA to examine the effects of *in vivo* NE administration on *in vitro* antibody responses to T-dependent and T-independent antigens. They reported differential effects based on the thymus dependency of the antigen and the timing of immunization relative to NE administration. Implantation of an NE-containing pellet prior to priming with a T-dependent antigen resulted in increased antibody production, whereas pellet implantation at some (unstated) time after priming was without effect. The influence of NE on antibody responses to T-independent antigens was studied by injecting NE rather than implanting an NE-containing pellet. A single injection of an (unspecified) amount of NE at the time of immunization with TNP-LPS effected a reduction of the splenic anti-TNP antibody response. Unfortunately, the difference in routes of administration of the NE between the two experiments makes a general conclusion difficult. Addition of NE (10^{-6}–10^{-8} M) to cultures of splenocytes from animals primed *in vivo* with TNP-LPS also reduced the *in vitro* anti-TNP antibody response. Interestingly, an increase was seen if the cells were exposed to 10^{-12} M NE, a finding apparent from the data but not pointed out in the text. A low concentration (10^{-12}–10^{-15} M) of the β-AR agonist, isoproterenol, also enhanced *in vitro* antibody production when it was added on day 7 in culture. In contrast, the α2-agonist, clonidine (10^{-9}–10^{-15} M) resulted in a reduced response, an effect that was blocked by the α-antagonist, yohimbine. β-AR stimulation with 10^{-10}–10^{-12} M isoproterenol was also reported to reduce *PHA*-stimulated T-cell mitogenesis, a finding which we were unable to replicate using physiological or even subpharmacological (i.e., less than 10^{-4} M) concentrations of isoproterenol (Cohen and Kinney, unpublished). Although Clothier and colleagues (1992) also claimed that immunization resulted in a depletion of NE in the spleen, an unexplained decrease in splenic NE was also seen in animals injected only with phosphate buffered saline (PBS).

In these same studies, Clothier et al. (1989, 1992) suggested reciprocal interactions between splenic NE content and processes involved in antibody production. Treatment with 6-OHDA reduced primary anti-hapten antibody responses to TNP-SRBC. When TNP was coupled to a T-independent carrier (LPS or Ficoll), the primary anti-hapten response was increased.

Most recently, Haberfeld et al. (1999) reported that adrenoceptor agonists modulate *in vitro* apoptosis of lymphocytes. Although α-2 and β-2 receptor agonists themselves did not induce apoptosis of *Xenopus* splenocytes cultured for 4 or 20 hours, they did modulate apoptosis of *Xenopus* splenocytes that were cultured with a calcium ionophore. More specifically, clonidine (α-2 agonist) enhanced ionophore-induced apoptosis of lymphocytes cultured for 4 but not for 20 hours, whereas isoproterenol (β-2 agonist) decreased apoptosis in 4-hour cultures but enhanced this programmed cell death when lymphocytes were cultured for the longer period. By itself, the synthetic glucocorticoid, DEX (10^{-4}–10^{-6} M), also induced apoptosis of frog lymphocytes cultured for 4 or 20 hours. Whereas clonidine did not affect cell death caused by this synthetic steroid at either time-point, isoproterenol enhanced apoptosis after 4 but not after 20 hours of co-culture. Haberfeld et al. (1999) suggested that their data might reflect a different apoptotic pathway induced by the ionophore and the synthetic steroid.

All the aforementioned experiments dealing with noradrenergic modulation of immunity in amphibians were based on the assumption that the agonists and antagonists used were actually binding to bona fide β-AR receptors. Józefowski and Plytycz (1998) used radiolabeled ligand binding to actually characterize β-AR receptors on splenocytes and activated peritoneal leukocytes from frogs (*R. temporaria* and *B. bufo*) and splenocytes from the salamander (*Salamandra salamandra*). Saturation, competition, and kinetic studies revealed a single site with a similar binding capacity. These investigators estimated that there were about 4,000 and 14,000 receptors per frog splenocyte and peritoneal cell, respectively (the difference probably reflects the larger number of phagocytic adherent cells in the peritoneal exudate) and as many as 183,000 such receptors on salamander splenocytes (a number consistent with their greater cell size). Although leukocytes from amphibians and goldfish (see previous section) all have β-AR receptors with similar affinities for the ligands [^{3}H]CGP-12177 and [^{3}H]DHA, competition experiments (Józefowski and Plytycz, 1998) suggest that there may be taxa (class)-specific differences not only for cells of the amphibians and teleosts

[2]To stimulate α receptors, either the specific α-agonist, phenylephrine, or a combination of epinephrine plus the β–receptor antagonist, timolol, were used; to stimulate β–receptors, either the specific β agonist, isoproterenol, or a combination of epinephrine plus the α–receptor antagonist, thymoxamine, was used.

studied, but also relative to birds and mammals. Radiolabeled ligand-binding studies have also revealed cholinergic muscarinic receptors on elicited peritoneal leukocytes from two species of anurans (Józefowski et al., 1996).

As discussed in great detail in the first section, which deals with invertebrates, a series of studies published throughout the 1990s by Ottaviani and coworkers used immunocytochemical procedures to investigate whether neuropeptides, neurohormones, and cytokines are produced by vertebrate and invertebrate cells and tissues involved in adaptive and innate immunity. With respect to the Amphibia, POMC-derived ACTH, β-endorphin, and α-melanocyte–stimulating hormone (α-MSH), as well as molecules that show antigenic cross-reactivity with antibodies directed against mammalian IL-1α, IL-1β, IL-2, IL-6, and TNF-α were revealed in PAS-positive epithelial cells of the thymus of the anuran amphibian *R. esculenta*. Three groups of PAS-positive epithelial cells were identified in subcapsular cortex, inner cortex, and medulla. The cells containing ACTH-, α-MSH- and cytokine-like molecules were distributed in the cortex, whereas those containing β-endorphin–like molecules were found in the medulla and inner cortex. Thymic lymphocytes were always negative for POMC-derived peptides and "cytokines." This does not appear to be the case for peripheral lymphocytes, however, since immunocytochemistry and cytofluometry (Ottaviani et al., 1992c, 1995a) detected POMC (or POMC mRNA) in peripheral blood lymphocytes as well as in phagocytic leukocytes from this same species. Ottaviani et al. (1998) also detected CRH and cortisol-like molecules immunocytochemically in the epithelial cells, interdigitating cells and macrophages, but not lymphocytes, in thymuses from fish, frog, chicken, and rat. These data suggest that throughout vertebrate evolution, the thymus can function as a neuroendocrine organ that is fully capable of displaying characteristics of a "stress response." In a 1992 study, the presence of immunoreactive ACTH and β-endorphin molecules in phagocytic basophils and neutrophils of salamanders (*S. salamandra, T. carnifex, Speleomantes imperialis*) has been established (Ottaviani et al., 1992d); ACTH increased phagocytic activity of these cells.

Morphine-induced inhibition of zymosan-induced peritoneal inflammation consistently recorded in several mouse strains and in fish (salmon and goldfish) was not observed in the anuran amphibians *R. temporaria, R. esculenta,* and *Bombina bombina* (Kolaczkowska et al., 2000). Given the number of parameters involved in this type of study, however, a negative result cannot be considered as definitive.

In a more recent study, Chadzinka and Plytycz (2004) pre-incubated leukocytes from mice, goldfish, and frogs with agonists of mu, delta, or kappa opioid receptors (morphine, deltorphine, or U-50,488H, respectively), and then recorded their *in vitro* migration after culturing them in either medium (M), control serum (S), or serum from zymosan-treated animals (SZAS). In all species, migration of control leukocytes was in the order SZAS > S > M. Also for all species, pre-treatment of leukocytes with a mu or delta opioid receptor agonist, but not a kappa agonist, enhanced their migration when they were cultured with either medium or control serum. When the leukocytes were harvested from animals treated *in vivo* with zymosan to activate them, the migration of mouse and fish leukocytes, but not frog leukocytes, was inhibited. This inhibition could be reversed by specific antagonists of mu and delta opioid receptors (CTOP and naltrindole, respectively). According to the authors, these results imply that the final effects of opioids on cell migration are "dependent on a species-specific balance between up- and down-regulation of leukocyte migration that results from an interplay between receptors for opioids and chemotactic factors."

C. Seasonal Influences on Immunity

The amphibian immune system, like that of reptiles (see next major section), is influenced by seasonal variations (Plytycz and Seljelid, 1996, 1997). Several examples are worth noting: (1) The inguinal lymphoid bodies of toads undergo seasonal cyclic changes in morphology (Plytycz and Szarski, 1987). (2) Gut-associated lymphoid tissues of some anuran species also undergo seasonal changes (Saad and Plytycz, 1994). For example, Wojtowicz and Plytycz (1997) found that the number of lymphoid nodules in the gut was negligible in field-collected *B. bufo* that were emerging from hibernation. This number increased in spring, reached its highest level in the summer, and declined in autumn. (3) The magnitude of the anti-SRBC response and the number of splenic lymphocytes in the toad *B. regularis* are high in spring, low in summer, high in autumn, and low again in winter (Hussein et al., 1984; Saad and Ali, 1992). (4) The percentage of lymphocytes in peripheral blood and hematopoiesis in the perihepatic subcapsular tissue is strikingly reduced in winter relative to summer in two species of newts: *T. carnifex* and *T. alpestris* (Barni et al., 1993). (5) The thymus of the frog *R. temporaria* undergoes cyclical changes with maximal development occurring during the summer and involution in the winter (Bigaj and Plytycz, 1984; Miodonski et al., 1996; Plytycz, et al., 1991).

Surprisingly, only a few investigators have examined seasonal effects on the amphibian immune system by modeling the normal winter hibernation of amphibians in the laboratory. In an early study of this kind, Green (Donnelly) and Cohen (1977) found that complement CH50 titers of *Rana pipiens* kept at 4°C for several months in a hibernaculum decreased to undetectable levels. This finding was confirmed and extended to other components of the immune system by Maniero and Carey (1997). Cooper et al. (1992) performed a more comprehensive study of hibernation-associated changes in the anuran immune system. Leopard frogs (acclimated to 22°C) were placed in a hibernaculum (in the dark) in late autumn. Animals, sampled at days 45 and 90 after initiation of hibernation, underwent a progressive 9- to 10-fold loss of leukocytes in the blood, thymus, spleen, jugular bodies and, to a much lesser extent (1- to 2-fold), in the hematopoietic bone marrow. These changes were reflected by marked aplastic changes in these organs. By the end of the experimental hibernation period (day 135), however, numbers of leukocytes in these organs had begun to increase, even though the photoperiod was unchanged and the temperature remained at 4°C. At day 30 after the frogs had been returned to room temperature, cell numbers had returned to, or were greater than, pre-hibernation values, and the architecture of the organs again appeared normal.

Despite the clear importance of temperature effects rather than season on graft rejection discussed below, at least in the case of the *R. temporaria* thymus, manipulation of the environmental temperature during the period of winter involution and summer growth did not override the endogenous seasonal rhythms (Bigaj and Plytycz, 1984; Zapata et al., 1992). These morphological and functional changes may result from seasonal changes in levels of circulating hormones such as corticosteroids, sex steroids, GH, PRL, and/or thyroxine (Mosconi et al., 1994; Plytycz et al., 1993; Saad and Ali, 1992; Zapata et al., 1992). Indeed, nearly 60 years ago, Holzapfel (1937) reported changes in the gross and histological changes in the adrenals, pituitary, gonads, pancreas, and thyroid during winter hibernation.

This yearly pattern of thymic involution and loss of lymphocytes followed by expansion of thymocytes and peripheral lymphocytes in the spring may offer a unique model for studying repertoire development and self-tolerance in the context of neuroendocrine regulation. Regardless of whether cold and/or neuroendocrine changes are responsible for the striking winter-associated depression of lymphoid tissues and specific immune responses (Plytycz and Seljelid, 1997), immunologists still have to contend with the issue of how such immunosuppressed amphibians survive during such periods. Several possibilities come to mind. First, at least some animals might not survive. Second, at least for amphibians living in temperate climates, low temperature may also suppress the growth of potential pathogens. Third, immunological memory (i.e., memory cells) to pathogens to which amphibians were exposed during the spring, summer, and fall may persist during the winter (Cooper et al., 1992). Finally, components of the non-specific innate immune system may not be depressed during the winter or may recover more rapidly after hibernation than specific adaptive immune responses. With respect to this last possibility, Maniero and Carey (1997) noted that complement activity increased within 2 days after *R. pipiens* were brought from artificial hibernation to room temperature. Moreover, complement levels continued to increase until, in some instances, they were significantly greater than those of controls held at room temperature. Similarly, Plytycz and Józkowicz (1994) reported that endocytosis of peritoneal macrophages from fish as well as anuran and urodele amphibians was effective *in vitro* over a wide temperature range and was actually enhanced in cells obtained from cold-acclimated animals.

Although the aforementioned studies reveal important seasonal (endocrine) effects on parameters of the amphibian immune system, it is well known that immune responses in this vertebrate class are, like those in other ectotherms, regulated by temperature. In this regard, it is noteworthy that the timing of skin graft survival by anurans grafted during different seasons appears reflective of temperature rather than neuroendocrine changes (Józkowicz and Plytycz, 1998; Saad and Plytycz, 1994). In the Józkowicz and Plytycz (1998) study, the effects of ambient temperature (22°C vs. 10°C) and season (summer vs. winter) on anuran skin allograft rejection were examined in frogs (*R. temporaria* and *R. esculenta*) and toads (*B. bufo, Bombina variegate,* and *B. bombina*). Mean graft survival times were significantly prolonged at the low temperature regardless of the season. Interestingly, *R. esculenta* was the most sensitive to the temperature effect, whereas *B. bufo* was relatively resistant. Rejection of second-set grafts in *R. esculenta* was accelerated at both 22°C and 10°C, but second-set grafts were less temperature-sensitive than the initial sensitizing ones. In both summer and in winter, *R. esculenta* rejected allografts promptly at 22°C but slowly at 10°C. In both seasons, *B. variegate* kept at 22°C rejected allografts chronically. This indicates that amphibian transplantation immunity depends on the donor-host genetic disparity and ambient temperature but is independent of season.

D. Glucocorticoid Effects on Amphibian Immunity

Exogenous administration of a single injection of DEX in *Rana perezi* caused thymic involution and massive destruction of cortical lymphocytes, diminution in white pulp, and lymphocyte redistribution to the bone marrow from blood and spleen (Garrido et al., 1987). The nature and extent of these changes depended on whether DEX was administered in autumn or winter (Garrido et al., 1989). Pharmacologic concentrations of hydrocortisone suppress antibody responses in *Xenopus* and cause massive cell death *in vivo* and *in vitro* in the thymuses and jugular bodies, but not the spleen, of *R. temporaria* (Plytycz et al., 1993). In the Plytycz et al. (1993) study, the number of viable cells in the thymus and jugular body returned to normal within 1 week after a single injection of hydrocortisone[3]. Rollins-Smith and Blair (1993) also reported that physiological (10^{-5} M–10^{-9} M) rather than pharmacological concentrations of corticosterone inhibited PHA-induced proliferation of adult *Xenopus* splenocytes.

E. Neuroendocrine-immune System Interactions During Metamorphosis

Thyroxine (T4) and triiodothyronine (T3) play a major role in driving amphibian metamorphosis (Kikuyama et al., 1993; Leloup and Buscaglia, 1977; White and Nicoll, 1981). Circulating levels of these hormones are low at pre-metamorphic stages, increase during pro-metamorphosis, peak during climax, and decline at the end of metamorphosis. Metamorphosis is also accompanied by increases in circulating levels of corticosteroids (Jaudet and Hatey, 1984; Kikuyama et al., 1986), PRL, and GH (Buckbinder and Brown, 1993; Clemons and Nicoll, 1977; White and Nicoll, 1981). Each of these hormones has immunomodulatory effects in mammalian species (Madden and Felten, 1995), and manipulation of at least some of these hormones also influences immune function in amphibians (Rollins-Smith, 1998; Rollins-Smith and Cohen, 1995, 2003).

Thyroid hormones: Since metamorphosis is strictly regulated by the availability of thyroid hormones, manipulating their levels makes it possible to inhibit or accelerate metamorphosis and thereby determine the extent to which development of an adult parameter of immunity in frogs requires a normal metamorphic transition. *Xenopus* larvae reared in the water containing the goitrogen, sodium perchlorate, to inhibit iodine uptake by thyroid follicle cells (Capen, 1994) undergo arrested development and do not metamorphose. Such blocked animals reveal that development of some structural and functional components of the adult *Xenopus* immune system are dependent on thyroid hormones and a normal metamorphic transition, whereas others are metamorphosis-independent. Specifically, perchlorate-blocked larvae lack the adult pattern of antibody responses to a specific hapten (Hsu and Du Pasquier, 1984), and adult-type MHC class II^+ T-lymphocytes do not appear in the periphery (Rollins-Smith and Blair, 1990b). Moreover, the thymus does not assume an adult-type morphology (Clothier and Balls, 1985); lymphocytes in the thymus and spleen do not achieve the expanded cell numbers characteristic of post-metamorphic adults (Rollins-Smith and Blair, 1990a, 1990b); adult-type skin allograft rejection does not replace the typical tolerogenic responses of larvae (Cohen and Crosby, unpublished); and, as pointed out previously, splenic innervation and compartmentation of the spleen into a clearly defined red and white pulp is dramatically delayed (Kinney, 1996).

Immune system changes that occur in perchlorate-blocked tadpoles (i.e., are independent of thyroxine levels) include the expression of MHC class I antigens (Rollins-Smith, 1998; Rollins-Smith et al., 1994), the immigration and expansion of T-cell precursors in the thymus (Rollins-Smith et al., 1992), and the development of high titer IgY antibody production (Hsu and Du Pasquier, 1984).

Just how thyroid hormones are related to the maturation of some facets of the *Xenopus* immune system is unknown. Thyroxine appears to drive the terminal maturation of larval erythrocytes (Galton and St. Germain, 1985) and the expansion of a separate adult erythrocyte population (Flajnik and Du Pasquier, 1988). However, since thyroid hormones have no deleterious effects on lymphocyte viability and inconsistent effects on proliferation (Rollins-Smith and Blair, 1993), the direct action of thyroid hormones does not appear to be responsible for the dramatic loss of larval lymphocytes at metamorphosis. In this regard, it is noteworthy that thyroid hormone deprivation initiated after metamorphosis does not appear to affect lymphocyte populations of post-metamorphic animals or the ability of T-cells from such animals to respond to mitogens (Rollins-Smith et al., 1993).

Corticosteroid hormones (CH): In short-term *in vitro* studies, both adult and larval lymphocytes are killed by concentrations of corticosterone that are comparable to those occurring at metamorphosis (Rollins-Smith, 1998; Rollins-Smith and Blair, 1993; Rollins-Smith et al., 1997a). Corticosterone and aldosterone signifi-

[3]It should also be noted that thymic and splenic morphology may be affected by laboratory and/or husbandry conditions regardless of season (Dulak and Plytycz, 1989).

cantly inhibit PHA-induced proliferation of larval and adult spleen cells (Marx et al., 1987; Rollins-Smith and Blair, 1993; Rollins-Smith and Cohen, 1995), and corticosterone induces apoptotic death of larval splenocytes at concentrations as low as 1–10 nM (Rollins-Smith, 1998; Rollins-Smith et al., 1997a). Adult lymphocytes may be protected *in vivo* from the destructive effects of corticosterone since there is less total corticosterone in the circulation after metamorphosis, and most is in a bound state (Jolivet-Jaudet and Leloup-Hatey, 1986).

Since aldosterone levels do not appear to be affected by specific serum-binding factors, the observed elevated levels of aldosterone in plasma of metamorphosing and adult frogs reflect the level of freely available aldosterone. Because larval lymphocyte function is affected by aldosterone at these physiologically relevant concentrations (Rollins-Smith and Cohen, 1995), aldosterone could play a role in the loss of larval lymphocytes.

Based on *in vitro* sensitivities of *Xenopus* lymphocytes to corticosterone and aldosterone, it is reasonable to propose that the naturally increasing concentrations of these hormones during metamorphosis are causally related to the death of significant numbers of larval splenic lymphocytes (Rollins-Smith and Cohen, 1995). This hypothesis is supported by evidence that the *in vitro* inhibition of PHA-stimulated splenocyte proliferation by CH can be blocked by the CH-receptor antagonist, RU486 (Rollins-Smith, 1998; Rollins-Smith and Cohen, 1995; Rollins-Smith et al., 1997a), and that *in vivo* treatment of stage 57–58 larvae with RU486 reveals a dose-dependent inhibition of the loss of splenocytes that normally occurs during metamorphosis (Rollins-Smith, 1998). Thus, corticosterone and/or aldosterone appear to be important regulators of peripheral splenocyte populations at metamorphosis in *Xenopus.* The putative hormonally induced loss of splenic lymphocytes during metamorphosis is quite important, for if these immunocompetent larval cells were not eliminated, they could potentially be activated by newly emerging adult-specific self-molecules.

The role of increasing levels of CH on the viability and development of thymocytes is more difficult to evaluate. Viability of larval thymocytes cultured in 1–10 μg/ml corticosterone is significantly reduced (Rollins-Smith and Blair, 1993); this reduction can be inhibited by RU486 *in vitro* (Rollins-Smith, 1998; Rollins-Smith et al., 1997a). However, other studies have reported significant apoptosis of cultured thymocytes from all larval and metamorphic that does not appear to be increased by culture with DEX (Ruben et al., 1994). Regardless of their outcome, these *in vitro* studies are difficult to interpret because thymocyte development is critically dependent on influences of the stromal/epithelial microenvironmental. For example, in mammalian systems, thymic epithelial components can synthesize corticosteroids (Vacchio et al., 1994). Thus, thymocytes may constantly receive local corticosteroid signals that influence their viability.

Growth hormone (GH) and prolactin (PRL): Although a role for GH and PRL in mammalian immunity is now well established (Dorshkind and Horseman, 2000; Kelly et al., 1992), very little is known about the involvement of these hormones in the development and function of cells of the amphibian immune system. Hypophysectomized tadpoles continue to grow, can usually reject skin or organ allografts differing by presumed or defined MHC antigens (Rollins and Cohen, 1980; Rollins-Smith and Cohen, 1982; Maéno, and Katagiri, 1984) and, like control tadpoles, do not reject minor H-locus disparate grafts or grafts expressing organ-specific antigens (Maéno and Katagiri, 1984; Rollins-Smith and Cohen, 1982).

To study the possible role of pituitary-derived hormones in the development of the amphibian immune system, Rollins-Smith and colleagues (2000) hypophysectomized (hypx) early larval stage *Xenopus* and examined the pattern of immigration of host-derived T-cell precursors into an implanted thymus using a ploidy marker (Rollins-Smith et al., 1992; Turpen and Smith, 1989). There were significantly decreased numbers of lymphocytes in the spleen and thymuses of hypx frogs in comparison with intact controls. However, no apparent delay or inhibition of the repopulation of the triploid donor thymus implanted into a diploid tadpole host was observed (Rollins-Smith et al., 2000). These results suggest that during the larval period, pituitary hormones are not required for either maintenance of the extra-thymic stem cell compartment, for maintenance of the attractiveness of the thymus for precursors, or for the process of thymocyte immigration. Analysis of cell division in the developing thymus populations revealed an inhibition of proliferation in hypx hosts. Thus, pituitary hormones appear to be required for the normal expansion of T-cell precursors after they colonize the thymus.

In unpublished studies, we and Rollins-Smith have independently observed that chicken anti-PRL antibodies suppress proliferation of mitogen-stimulated (LPS, PHA, Con A) larval and adult splenic lymphocytes. Thus, PRL may be an essential lymphocyte growth factor that developed early in vertebrate evolution (see discussion in Kelly et al., 1992). To produce sufficient *Xenopus* PRL to investigate the role of PRL in normal lymphocyte function, Rollins-Smith and