

Effects of Infection with the Monogenean *Neoheterobothrium hirame*
on the Survival of Wild Olive Flounder *Paralichthys olivaceus*

単生類 *Neoheterobothrium hirame* の寄生が天然ヒラメの生存に及ぼす影響

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論文の内容の要旨

1. 序論

ヒラメは日本の沿岸漁業並びに養殖業にとって重要な魚種であり、その資源増大を目的として各地で放流事業が行われている。しかし、90年代半ばより一部海域で漁獲量が急減し深刻な問題となっている。顕著な例では鳥取県で過去5年間に漁獲が80%も減少しており、沿岸漁業および放流事業への影響も出ている。このヒラメ漁獲量の減少と同時期に単生類 *Neoheterobothrium hirame* (以下、本虫) の寄生による重篤な貧血症状を呈したヒラメが目立ち始めた。これまでの研究から本虫は外国からの移入病原体であると考えられている。

ヒラメ漁獲量の減少がみられている西部日本海では本虫の寄生率が高く、本虫の寄生による貧血が高頻度に見られる。また、その他の海域でも本虫の出現時期と漁獲量減少の時期が一致することなどから、本虫の寄生が天然ヒラメ資源減少の原因であることが示唆されている。本虫の寄生の有無と各海域での漁獲量減少が必ずしも一致しないことから、本虫と資源減少の関係は少ないとする見方もある。しかし、これまで両者の因果関係についての研究は断片的で、その解明を目指した総合的な研究は行われていなかった。そこで、本研究はこの因果関係を明らかにすることを目的とした。まずヒラメ漁獲量の減少と本虫の寄生状況の関連を把握するため、漁獲量の減少がみられる海域と漁獲量が比較的安定している海域間で本虫の寄生動態を調べた。これによって得られたデータから、寄生数には冬季の低水温が大きな影響を及ぼしていると考え、低水温が本虫に与える影響を実験的に調べた。さらに、本虫の寄生が間接的にヒラメ稚魚の死亡に関与しているとの仮説を検証するため、実験感染させたヒラメ稚魚の活動パターン、潜砂行動、遊泳行動、被食、摂餌行動、飢餓耐性、およびウィルス性疾病への感受性を調べた。

2. 天然調査による寄生動態の比較

ヒラメ漁獲量の減少は西部日本海で顕著であるが、北部太平洋岸での漁獲量は安定している。そこで、これらの海域における本虫の寄生動態を比較した。2002年－2004年に、福井県小浜湾と岩手県宮古湾から毎月ヒラメ（0－1才）を採集し、寄生状況ならびに寄生虫の発育状態を調べたところ、寄生数は秋に急増し、12－2月に最高値に達した後、減少するという冬高夏低の傾向がみられた。両海域の寄生率はともに90%に達したのに対し、寄生数は小浜湾の方が4倍近く高い月もあるなど、両海域で顕著な差がみられた。また、寄生虫の発育段階を月毎に調べた結果、0才魚に寄生がみられはじめてから寄生数が最高値に達する約半年間の本虫の感染サイクルは、小浜湾では3－4回、宮古湾では2回程度であることが示唆された。

これらの結果から漁獲量の減少がみられる海域では寄生数が高いことが明らかになり、寄生数調査の重要性が示唆された。また海域によって本虫の寄生動態が異なるため、何らかの海域特性が本虫の増減に影響しているのではないかと考えられた。

3. 低水温下における本虫孵化幼生の着定とその発育

本章では、前章で明らかになった寄生動態の違いの原因を明らかにするため、両海域における違いが顕著で、本虫に対する影響が大きいと思われる水温に焦点を当てて実験を行った。小浜湾の年間平均水温は18℃と比較的温暖であるが、宮古湾では平均水温が10℃以下の月が年間4－5ヶ月ある。しかしこれまで、10℃以下の水温での本虫の発育等は調べられていなかった。

低水温下における本虫の感染力を調べるため5℃、10℃、20℃において6穴ウェルプレート内に置いたヒラメ鰓片への孵化幼生の着定を24時間観察したところ、水温と着定数には相関がみられ、5℃では20℃の30%程度の着定しかみられなかった。また本虫を寄生させたヒラメ稚魚を8℃と20℃で105日間飼育し、虫体の発育を定期的に観察した結果、8℃で

は著しい成長の遅れと未成熟虫数の減少がみられ、低水温により本虫が死亡又は脱落したと推察された。これらの結果から、低水温は本虫の寄生に対する阻害要因となることが示唆され、宮古湾における低い寄生数は冬季の低水温による影響が大きいと考えられた。

4. 寄生を受けた稚魚の行動と被食

これまで、天然ヒラメにおける減耗の多くは被食によると考えられている。本章では本虫の寄生によりヒラメ稚魚が被食されやすくなる、という仮説を検証するため、実験感染魚の行動並びに被食状況を非感染魚と比較した。

本虫を寄生させたヒラメ稚魚（体長約 10cm）を砂を敷いた水槽に入れ、各個体の潜砂率ならびに活発に遊泳をしている魚の割合を 1 時間毎に 24 時間観察し、対照区と比較した。その結果、感染魚は底砂からの体表露出面積が多く、非感染魚とは異なり午前 3 時をピークに夜中に活発に活動していることが分かった。さらに、回流水槽内でヒラメの持続遊泳時間を調べたところ、感染魚の遊泳時間は非感染魚に比べ著しく短かった。さらに、感染および非感染ヒラメ稚魚を水槽内で大型のヒラメ（捕食者）と同居飼育したところ、感染稚魚の被食率がより高いことが分かった。以上の結果から、本虫がヒラメ稚魚の行動に影響を与え、被食率を高めていることが明らかになり、天然の海でも本虫の寄生によってヒラメ稚魚の死亡が高まることが推察された。

5. 感染稚魚の摂餌と飢餓耐性

飢餓は天然海域においては被食と共に稚魚の減耗に深く関わっている要因の一つと考えられる。前章の実験で本虫はヒラメ稚魚の行動に影響を与えることから、本虫の寄生は摂餌行動にも影響を及ぼし、摂餌能力の低下を招いていると考えられた。さらに、本虫による吸血で、寄生を受けた稚魚では飢餓への耐性が低下することも想像され、本章ではこれらの仮説を検証するために実験を行った。

まず、感染魚と非感染魚 5 尾ずつを同時に入れた水槽に生きたイサザアミを投与し、摂食したアミ数を比較したが、両者の間に差はみられなかった。しかし、感染魚では摂食したアミの数と虫数の間に負の相関関係が認められた。次に、イサザアミに対する摂食行動を 30 分間ビデオ撮影し、比較した結果、感染魚は摂食行動中の離底時間が長く、遊泳高度も高い傾向にあることがわかり、捕食者からの攻撃をより受けやすいと考えられた。さらに、感染魚では捕食を伴わない離底行動もみられ、迷走型の遊泳が多い傾向にあった。一方、非感染魚では短距離しか移動しない瞬発型摂食行動が多くみられるなど両者の摂食行動パターンの差異が明らかになった。

最後に、重度感染と軽度感染のヒラメを作出し、非感染魚とともに 3 ヶ月間無給餌飼育してこれら 3 群の肥満度の推移や生存を比較したが、有意な差はみられなかった。

6. ウイルスとの混合感染

これまでの知見から、本虫の寄生はヒラメの直接の死亡原因とは考えにくいですが、寄生によって他の疾病への感受性が高まることは十分に考えられる。ウイルス性出血性敗血症ウイルス (VHSV) はこれまで天然魚からも確認され、養殖場等では大量死を引き起こしている病原性の強い病原体である。

そこで本虫に感染させたヒラメ稚魚に VHSV による攻撃した魚 (混合感染魚) について、その後の死亡を、本虫のみ感染魚、ウイルスのみ感染魚、非感染魚と比較したところ、混合感染区における死亡が最も高かった。しかしながら、水槽間による死亡の差が大きく、混合感染死亡魚の半数近くからはウイルスが検出されなかった。このことから、混合感染を受けたヒラメは死亡率が増加することは明らかとなったが、本虫感染が VHSV の感受性を高めるか否かは不明瞭であった。

これまでの研究から、VHSV 感染から生存したヒラメはキャリアになり、ストレスによって体内ウイルス量が再度増加することが分かっている。そこで、VHSV の筋肉注射後に生存

した魚に寄生虫を感染させ（混合感染区）、寄生虫のみ感染魚、ウイルスのみ感染魚、非感染魚との間で死亡数を比較した。その結果、混合感染区で最も死亡が多かったが、ウイルス感染区、寄生虫感染区にも死亡がみられた。しかし、死亡魚からウイルスは検出されなかったため、死因の特定には至らなかった。

7. 総合考察

本研究では天然調査と室内実験から、ヒラメ漁獲量が急減している海域では本虫の寄生数が多く、その傾向は水温に依存すること、本虫の寄生はヒラメ稚魚の行動に影響を与え、感染魚は被食されやすくなること、VHSV と本虫の混合感染はヒラメの死亡を高めることを明らかにした。これらのことから、当初の目的であった、本虫とヒラメ資源減少の因果関係を示唆する知見が得られた。しかし、宿主サイズ、寄生数と行動の変化との関係や、ウイルス感染実験における死亡原因など、今後さらなる調査が必要な課題も残った。

本研究では本虫の影響を稚魚でのみ検証したが、寄生が産卵行動や産卵数等ヒラメの生殖に及ぼす影響を確認することも重要であると思われる。また、本虫は流行直後からその動向が把握されており、天然ヒラメ調査の継続は移入寄生虫の動向を把握する上で重要である。近年の海外からの活魚、種苗、ペット等の輸入水生動物の増大に伴い、それらに付随してくる病原体による天然資源への影響が懸念されるケースが増えている。ヒラメのネオヘテロボツリウム症はこれら移入病原体による天然資源への影響を把握するモデルケースとして貴重かつ重要な研究課題であり、今後も引き続き長期モニタリングが必要であると考ええる。

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Chapter I. General Introduction

General Biology of Olive Flounder

Olive flounder, *Paralichthys olivaceus*, also known as Japanese flounder or bastard halibut, belongs to the family Paralichthyidae and is the only species of genus *Paralichthys* found in eastern Pacific (Minami, 1997). There are 19 known *Paralichthys* species worldwide, but most of their geographical distributions concentrate on the Pacific and Atlantic sides of the continents of North and South America. The distribution range of *P. olivaceus* is limited to western Pacific Ocean from Sakhalin, Kuril Islands, Japan, Korean Peninsula, to South China Sea. In Japan, *P. olivaceus* can be found throughout the coast, except in Okinawa and on the Pacific coast of northern Hokkaido (Minami, 1997). Although some of the past studies indicated the morphological and genetical differences among the flounder populations in Japan, the existence of complete genetical isolation whether complete genetical isolation among the populations exist or not is unclear (Minami, 1997).

Olive flounder has a unique life history in which it undergoes extensive metamorphosis from a normal fish-like pelagic larval stage to a demersal flatfish. Spawning occurs mainly in spring, but can be in February in Kyushu or July in Hokkaido (Noichi, 1997). During the spawning season, breeding adults of 2 years or older migrate toward the shore and lay as many as ten million eggs. Spawning ground is chiefly in the shallow water with mud-sandy or fine gravel substrate. Flounder egg is pelagic and dispersed by oceanic current. Egg hatching period varies from 33 to 165 hours depending on the water temperature (Noichi, 1997).

Unlike the adult, larval flounder is pelagic and swim in upright position with a symmetrical body possessing eyes on both sides of the head. The main diet of larval flounder is various zooplanktons, such as Appendicularia. During the development, larvae migrate toward shallow inshore nursery ground for settlement. This is the

period when the fish face drastic change in their biology. During the metamorphosis, the right eye gradually migrates across the dorsal side and to the left side of the head, and the body becomes laterally asymmetry and flattened. These changes occurs within one month after hatching. As the body become flattened, fish settle onto the bottom of the sea and become demersal. Development of gill rakers, scale formation, and pigmentation of ocular side take place following the settlement (Tanaka, 1997). Density of larvae-juvenile flounder is quite high during the settling period and this is the time when the major loss of fish occurs. Metamorphosis and the complete change in their life history is the great stress to the fish. Also, newly settled larvae are highly susceptible to the predation and only the limited proportion of larvae can survive this period. Post-larval flounder feeds predominantly on small crustaceans including various species of mysids. Their main diet gradually shifts to the small fish and they become a piscivorous predator. The timing of diet shift varies, but mostly occurs when fish are around 10 cm in the length (Noichi, 1997). Corresponding to the diet shift, juvenile fish leave the nursery ground and disperse toward the deeper water where they develop into mature adults.

Flounder is generally considered as a coastal resident fish that tends to stay in a limited area. However, mark and recapture studies have revealed that flounder are capable to travel quite a long distance. For instance, fish released in Wakasa bay, Tottori prefecture, travelled west over the Shimane peninsula to Shimane prefecture (Minami, 1997). In another case, a fish released on the Pacific side of Aomori prefecture was captured on the Sea of Japan side (Minami, 1997). The lifespan of flounder is normally within 10 years and they can grow up to 100 cm in length or 9 kg in body weight.

Flounder Fisheries and Resource Management in Japan

Olive flounder is one of the most popular white-meat fish in Japan. Japanese are

fond of its low-fat, light, yet elegant taste and it has been considered as one of the best fish for *sashimi* and *sushi* dish. As flounder is often consumed uncooked, many are traded as live. The average market value is around 1,500 yen per kg, but can be as high as 4,000 yen for a fresh, live wild ones (MAFF). Flounder fishing is mainly coastal and fishermen capture them by gill net, set net, or trolls. According to the statistical data from the Ministry of Agriculture, Forestry and Fisheries of Japan (MAFF), a total flounder catch in Japan has been approximately 7,000 t for the last several years. The amounts of annual catch fluctuate periodically over the last fifty years with the intervals of 10 - 15 years (Fig. I-1).

Flounder is also one of the most extensively cultured fish species in Japan. Flounder cultivation started in the mid 1970s and became increasingly popular during 1980s. In the 1990s, flounder productions in the aquaculture increased by 10-fold (Fig. I-1). Flounder culture reached its peak in 1997 with the total annual production of over 8,500 t. However, the production has been on decline since and it lays somewhere around 6,500 t in the past few years (statistics from MAFF). Flounder farming has started as a mariculture, but cultivation in a land-based tank is considered more efficient and has become a mainstream in today's farming.

As consumer's demand and fishing pressure for flounder increase, resource management is becoming an important issue. Flounder is one of the earliest fish species that was succeeded in seed production. The first production of flounder seedling was achieved in 1965 at Kinki University. Since then, serious efforts have been spent to develop efficient and effective techniques for seedling production, and stocking. Seedling production of flounder increased enormously over the last two decades and the total annual production reached over 40 million fish by year 2000 (Statistical data from The Japan Sea-Farming Association). Most of the seedlings were released into the water to sustain healthy stock of wild flounder. Stock enhancement program for flounder was started in 1970s and has become common

activities in national and municipal bodies throughout Japan. In 2002, over 25 million seedlings were released at nearly 2,000 releasing points throughout Japanese coast (The Japan Sea-Farming Association) (Fig. I-2).

Despite such diligent efforts, sharp drops in flounder catch have been noticed in some regions and have become serious concern. The decline of flounder catch is the most evident in south-western coast of the Sea of Japan. In Tottori prefecture, the annual catch has reduced to 20% of what used to be in 5 years ago, and this resulted in the termination of stock enhancement program (Fig. I-3). Similar crashes in commercial catch have also been observed along the coast of the Sea of Japan, Kyusyu, and also on the southern Pacific Sea. As the fishing effort has not been changed, this sudden decline of catch is considered to be resulted from the reduction of local flounder populations.

Population decline can be a consequence of over-fishing, pollution, habitat destructions, climate changes, food depletion, disease outbreaks, and/or simply a part of natural fluctuation. Diminution of fishery resources resulting from over-fishing in association with advanced fishing technology has become serious problems in the last 50 years in worldwide. For instance, heavy fishing pressure and destruction of ocean bottom by trawling has pushed the stocks of Atlantic cod to depletion (Erwan *et al.*, 2003). Other factors have been shown to influence the fishery resources. Global warming and other climatic events, such as El Niño, have been reported to affect marine ecosystem (Harvell *et al.*, 1999). The collapse of the Peruvian anchovy (*Engraulis ringens*) industry in the early 1970s was considered as a direct result of the El Niño (Ñiquen and Bouchon, 2004). Outbreaks of emerging diseases associated with climate changes and human activities also threaten many marine lives (Harvell *et al.*, 1999). Natural fluctuation also causes periodical decline of fish populations. However, in general, population of pelagic fish are more amenable to the natural fluctuation than that of demersal fish which tend to be influenced more by over-fishing

and habitat distraction (Ichiro Aoiki, personal communication).

Fishing pressures for flounder by both commercial and recreational fishing have been increasing over the past years. Constructions and exploitations of coastal areas changed the marine ecosystem and surrounding environments (Islam and Tanaka, 2004). All of these factors could have significant influence on flounder populations in Japan. However, any of these factors do not seem to be an ultimate cause for the sudden decline of flounder resources.

Concerning the over-fishing of flounder, local fishermen and municipals actively establish the catch limit, in addition to the extensive stocking efforts (Kitada *et al.*, 1992; Masuda and Tsukamoto, 1998). However, these resource management acts could not effectively restrain the reduction of fish catch. Moreover, decrement in the catch in some regions started even after the resource management acts have taken places. Hence, over-fishing is not likely a sole cause for the recent decline of flounder resource. Local constructions would not cause the depletion in such a wide geographical area. Periodical climate change may cause the fluctuation of the flounder population, yet, the reduction seen in Tottori seems to be extreme and beyond the range of the natural fluctuation (Fig. I-3). Although it is controversial whether the current decline of flounder population is substantially below the range of natural fluctuation, some researchers and fishermen have been starting to suspect other causes which explain this phenomenon better.

Anaemic Flounder and Invaded Parasite, Neoheterobothrium hirame

Sometime around 1995, flounder exhibiting severe anaemic symptoms, mainly discoloration of the gills and pale body colouration, has started to be noticed in the Sea of Japan (Miwa and Inoue, 1999). Reports of anaemic flounder were initially restricted from the Sea of Japan but the disease spread rapidly (Ogawa, 2002). By 1998, flounder with similar symptoms have been found on the other part of the Sea of

Japan as well as from the Pacific coast. Soon after, the diseases expanded its reach to fish farms and hatcheries throughout Japan (Michine, 1999). Researchers started to suspect the causal relationship between the epidemic of this anaemia and the decline of the flounder population. Researchers immediately took the action to identify the causative agent of the disease. Miwa and Inoue (1999) reported the presence of virus-like particles in the kidneys and spleens of anaemic flounder, thus a hitherto unidentified virus was first suspected as the causative agent. However, injection with blood or tissue homogenate did not reproduce anaemia, thus involvement of other pathogens was suspected (Tanaka *et al.*, 2001).

Coincided with the appearance of anaemic fish, a large monogenean parasite has been found in the buccal cavity of flounder (Michine, 1999; Ogawa, 1999). The parasite was first recorded in 1995 from the fish caught in the Sea of Japan, and soon later, the same parasite has been found in wild, farmed and stocked fish in various localities. Despite of its large body size and highly visible infection site, the parasite has never been reported previous to 1995. By tracing back the old stored flounder samples, the same parasite was found from the fish caught in 1993. Ogawa (1999) described this parasite as a new species of a diclidophorid monogenean *Neoheterobothrium hirame*, a similar parasite to *N. affine* Linton, 1898 of summer flounder, *P. dentates*, in North America.

Researchers have experimentally infected *N. hirame* to healthy flounder and successfully demonstrated of the anaemic symptoms (Tanaka *et al.*, 2001; Yoshinaga *et al.*, 2001a,b). In addition, infected flounder have been shown to recover from anaemia following the deworming procedure. It was then concluded that *N. hirame* is the causative agent of anaemia observed in wild flounder.

Since the first report in the Sea of Japan, *N. hirame* has stretched its geographical range at alarming rate. Today, the parasite has become ubiquitous throughout Japanese waters, and has also been found in Korea (Ogawa, 1999; Hayward *et al.*,

2001; Mushiake *et al.*, 2001). The prevalence of infection can be as high as 100% in some regions (Anshary *et al.*, 2002). Because of its sudden emergence, *N. hirame* is considered as a non-indigenous pathogen that recently invaded Japanese water. Recent molecular study further supports this, and suggested the parasite is originated from North America (Tsutsumi, 2004).

The life cycle of *N. hirame* is somewhat unique for Monogenea (Fig. I-4). An adult worm can be as large as 30 mm in length and inhabits in the buccal cavity of flounder, embedding its posterior part into the host tissue (Anshary and Ogawa, 2001). Normally, worms are found on the upper wall of the buccal cavity and several individuals are often found embedding their posterior end into the same locations to form a worm cluster. The adult elongates its extremely elastic body to feed the host's blood from the gill. One adult lays as many as 800 eggs per day (Tsutsumi *et al.*, 2002). Eggs are approximately 0.5 mm in length and possessing the appendages at the two ends, possibly to prevent from drifting away on the sand. When eggs are released, they sink in the sea water, settle on the bottom, and hatch within 5 to 10 days depending on the temperature (Yoshinaga *et al.*, 2000a). Emerged oncomiracidium, a free-swimming larva, seeks for the host and attaches to the body surface or gills of the next host (Shirakashi, unpublished observation). After settling onto the gills, the worm grasps the gill lamella using its well-designed clamps, feeds on the blood and grows. Initially, the parasite inhabits primarily on the gill filaments but gradually migrates toward the buccal cavity through gill raker and gill arch (Anshary and Ogawa, 2001). During the development, the number of clamps increases from 0 to 4 pairs.

The parasite causes physical damage to the host via feeding the blood. Infected flounder becomes severely anaemic, characterised by low haemoglobin contents, abundant immature erythrocytes and abnormality of cytoplasm (Yoshinaga *et al.*, 2000b). A significant correlation between numbers of adult worms and severity of anaemia has been shown in the wild fish (Mushiake *et al.*, 2001). Moreover,

occasional necrosis at the infection site of adult worm and the surrounding tissue is also evident. It has also been said that anaemic flounder are more vulnerable to the handling and transportation, though no conclusive evidence has yet been provided (Tanaka *et al.*, 2001). The parasite density on a single flounder can be several dozens up to a few hundred in an extreme case (Yoshinaga *et al.*, 2000b; Mushiake *et al.*, 2001; Anshary *et al.*, 2002).

Despite the pathogenicity associated with infection, mass mortality of the flounder infected with *N. hirame* has not been reported in the wild fish. Although infected flounder seem to be less vigorous and have reduced appetite, mortality of captive infected fish are very low or practically nought (Ito, 2004). Considering the above facts, *N. hirame* infection is probably non-fatal, or at least flounder do not die from the infection alone. However, *N. hirame* could play an important role in host death if other factors are involved. In natural environment, numerous factors are intricately intertwined and in such a complex situation, this invasive pathogen may become detrimental to the host.

Effects of Parasite on Host Population

Historically, predation and competition are thought as two key biotic factors determining an animal population size. Today, symbiosis is also recognised as an important component in animal ecology. Both experimental and observational studies have shown that parasite reduce host density or even drive host population to extinction (Dobson and Hudson 1986; Scott and Dobson 1989; Minchella and Scott 1991; Hudson *et al.*, 1992, 1998).

Parasite-mediated regulation (*sensu* Scott and Dobson 1989) of host population has long been suggested. The theoretical model states that the host population density is a function of parasite density, if parasites affect fecundity and the survival of an individual host (reviewed by McCallum 1995; McCallum and Dobson 1995).

However, although parasite-mediated depressions of reproduction and survival (fitness) in host organisms have been reported, empirical evidence for their regulatory effects on population level is limited.

Some of detailed studies on parasite regulation on a host population have been conducted on freshwater and terrestrial vertebrate hosts. Lemly and Esch (1984) suspected that the seasonal mortality of juvenile bluegill sunfish (*Lepomis macrochirus*) was caused by a trematode parasite, *Uvulifer ambloplitis*, and conducted series of experiments. In their system, reduction of fish density following the peak of *U. ambloplitis* infection was observed in a pond. Through the laboratory experiments, they showed that the mortality of heavily infected juvenile increased when the water temperature fell below 10°C. Moreover, a significant negative correlation between the numbers of metacercariae and host's condition factor was observed. They, therefore, concluded that change in the bluegill density in the pond was caused by the *U. ambleoplitis*. In another study, population density of reindeer (*Rangifer tarandus plathyrynchus*) in northern Norway was shown to be controlled by *Ostertagia gruehneri*, a gastrointestinal nematode, by reducing the fecundity of females (Albon *et al.*, 2002).

Impacts of parasites to host population are often more articulated and much more severe when the pathogens are introduced to a new environment (i.e. naïve host). Most striking examples of invasive pathogens can be found among viruses and microparasites. Avian malaria introduced to Hawaiian Islands through mosquito vector drove several native forest birds to extinction and many to become endangered (Atkinson *et al.*, 1995). Recent decline of amphibian population in the continents of America and Australia is believed to be caused by the introduction of fungus *Batrachochytrium dendrobatidis* to those areas via translocation of infected frogs as pet (Hanselmann *et al.*, 2004). Recent epidemics by a herpesvirus causing mass mortalities of pilchard, *Sardinops sagax*, in South Australia is originated from frozen

pilchard imported as feeds for cultured tuna (see Harvell *et al.*, 1999). A more familiar example is a KHV (Koi Herpes Virus) epidemic recently causing mass mortalities in Japanese carp. KHV was likely to be introduced to Japan by translocation of live carps through commercial activities. These are only a part of a long list and these anthropogenic forms of pathogen invasion is termed as “pathogen pollution” (Daszak, 2000).

Less is known for such appalling effects of macroparasites, largely due to their relatively lower pathogenicity. However, mass mortality and population reductions in some fish are believed to be caused by invasions of macroparasites. *Gyrodactylus salaris*, an ectoparasite of salmonid, was accidentally introduced to Norwegian waters in 1970s caused a significant loss in Atlantic salmon fisheries. Salmon stocks in some Norwegian rivers are completely lost since its invasion. A nematode *Anguillicola crussus* is considered relatively harmless parasite of Japanese eel *Anguilla japonica* (Knopf and Mahnke, 2004). After this parasite was introduced to Europe in early 1980s through importation of Japanese eel for aquacultural purposes, it quickly found a new host, European eel (*A. anguilla*) and spread at an enormous rate among naïve native host populations. European eel populations are now facing serious depletion and the most suspicious suspect is this small parasitic worm. *N. hirame* is also believed as an introduced parasite and researchers and fishermen are keeping close eyes onto this small but threatening invader.

Aim of the Study

The fact that *N. hirame* epidemic and reduction in host catch started at around the same time suggests a causal link between the two phenomena. Moreover, the parasite causes severe anaemia to the host. High prevalence of *N. hirame* in the wild flounder also implies its influence on the flounder resources. Therefore, it can be hypothesised that *N. hirame* plays an important role in the recent reduction of flounder catch in

Japan. However, there are two main contradictions against this hypothesis: 1) the decline in the flounder catch is evident in only limited regions, even though *N. hirame* occurs throughout Japanese waters, 2) there is no report of mass mortality in wild flounder caused by *N. hirame*, and the mortality of infected fish is not commonly observed, at least under the captive condition. Up to this day, there has been no comprehensive study investigating the effect of *N. hirame* on the wild flounder. The ultimate goal of the present study is to determine the causal relationship between *N. hirame* and decline of flounder population by assessing its effect on wild flounder.

Anshary *et al.* (2001) suggested that *N. hirame* causes mass mortality of the 0-year flounder population in Tottori prefecture. However, their conclusion was based solely on field data and was strictly inferential. Experimental studies which enable to quantify the effects of the parasite are necessary to provide more solid evidence. Predation, starvation, disease, and environmental stresses are considered as key factors causing the depletion of juvenile flounder in the wild (Noichi, 1997). As *N. hirame* infection does not normally cause the direct mortality to flounder, indirect effects of *N. hirame* on the survival of wild juveniles in conjunction with these listed factors is considered. Various experiments were designed to test the hypothesis that *N. hirame* infection additively reduces the survival of juvenile flounder against predation, starvation and other diseases. In addition, field study was conducted to obtain the recent infection status of wild juvenile flounder and to determine the factors causing differences in the magnitude of parasite effects in different geographical regions. This study provides important and rare insight to understand the threat of an invaded parasite to an indigenous animal species.

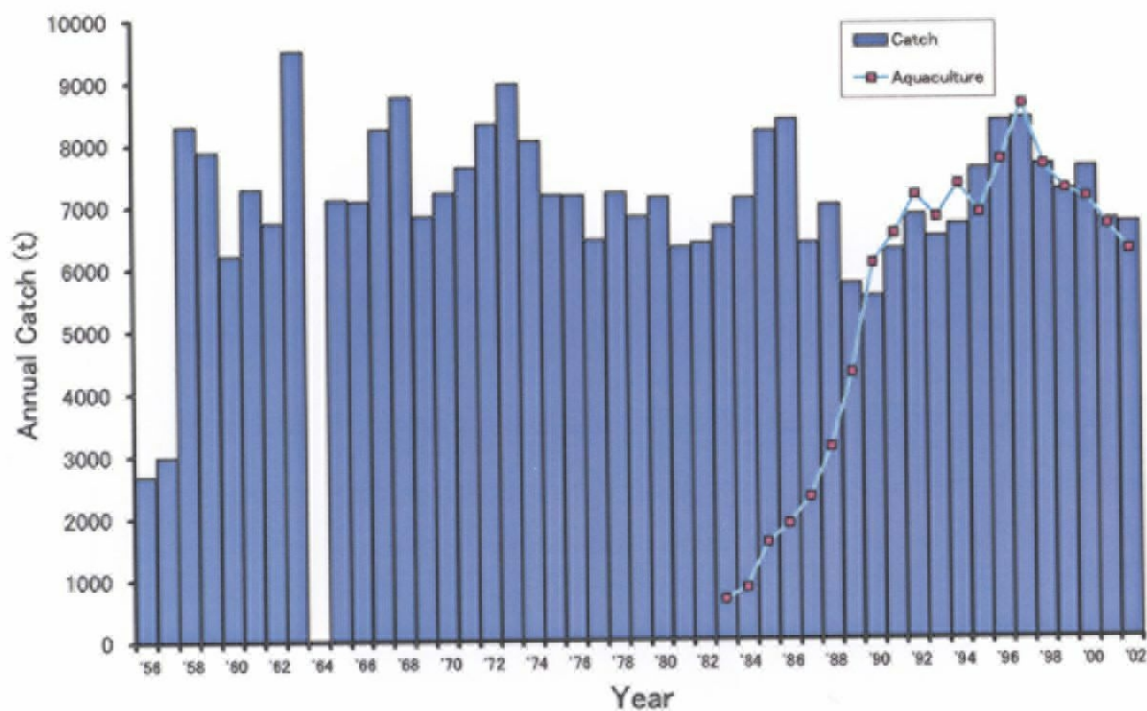


Figure I-1. Change in annual commercial catch and aquaculture production of olive flounder in Japan. Original data from Ministry of Agriculture, Forestry and Fisheries of Japan

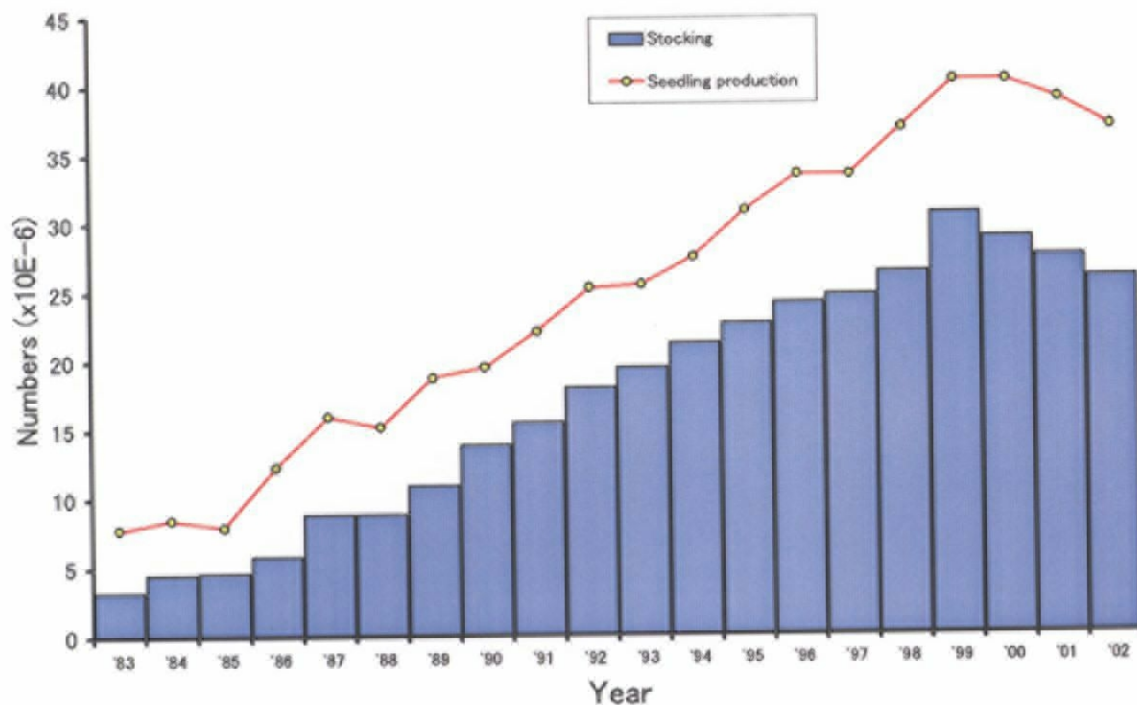


Figure I-2. Change in seedling production and numbers of released juveniles of olive flounder in Japan. Original data from Ministry of Agriculture, Forestry and Fisheries of Japan

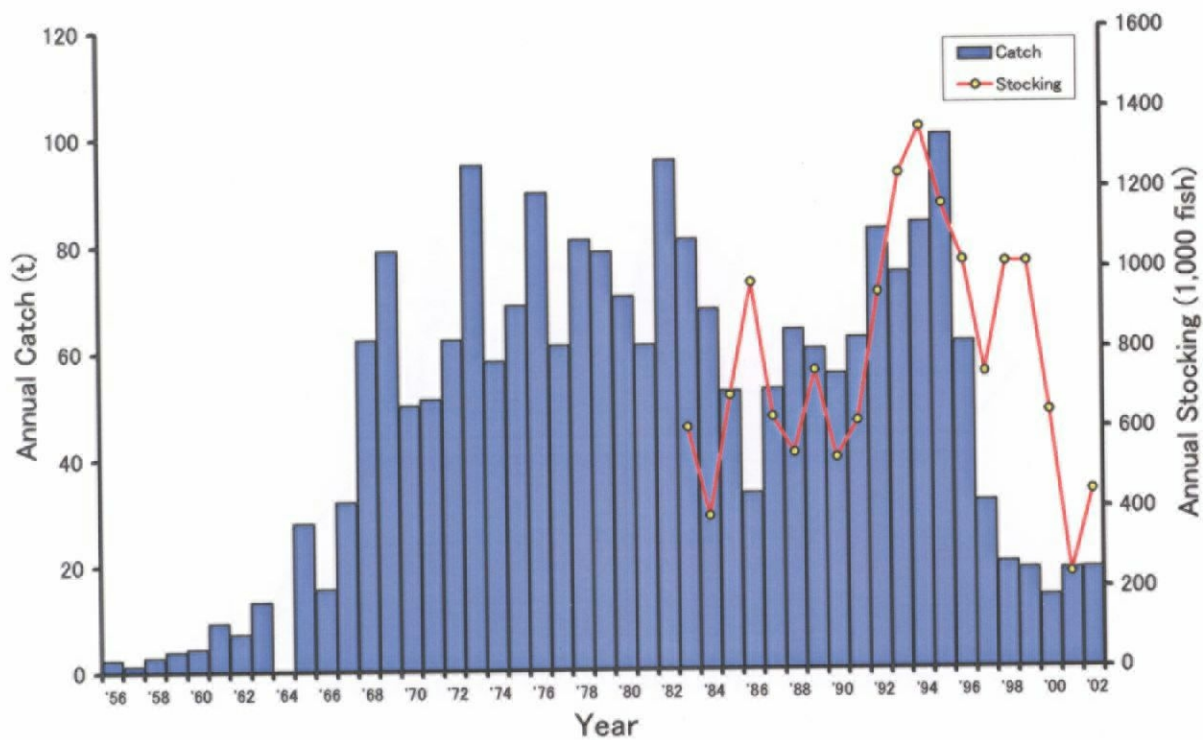


Figure I-3. Change in annual catch and stocking of flounder in Tottori prefecture. Note the sudden decline in annual catch after 1995. Original data from Ministry of Agriculture, Forestry and Fisheries of Japan

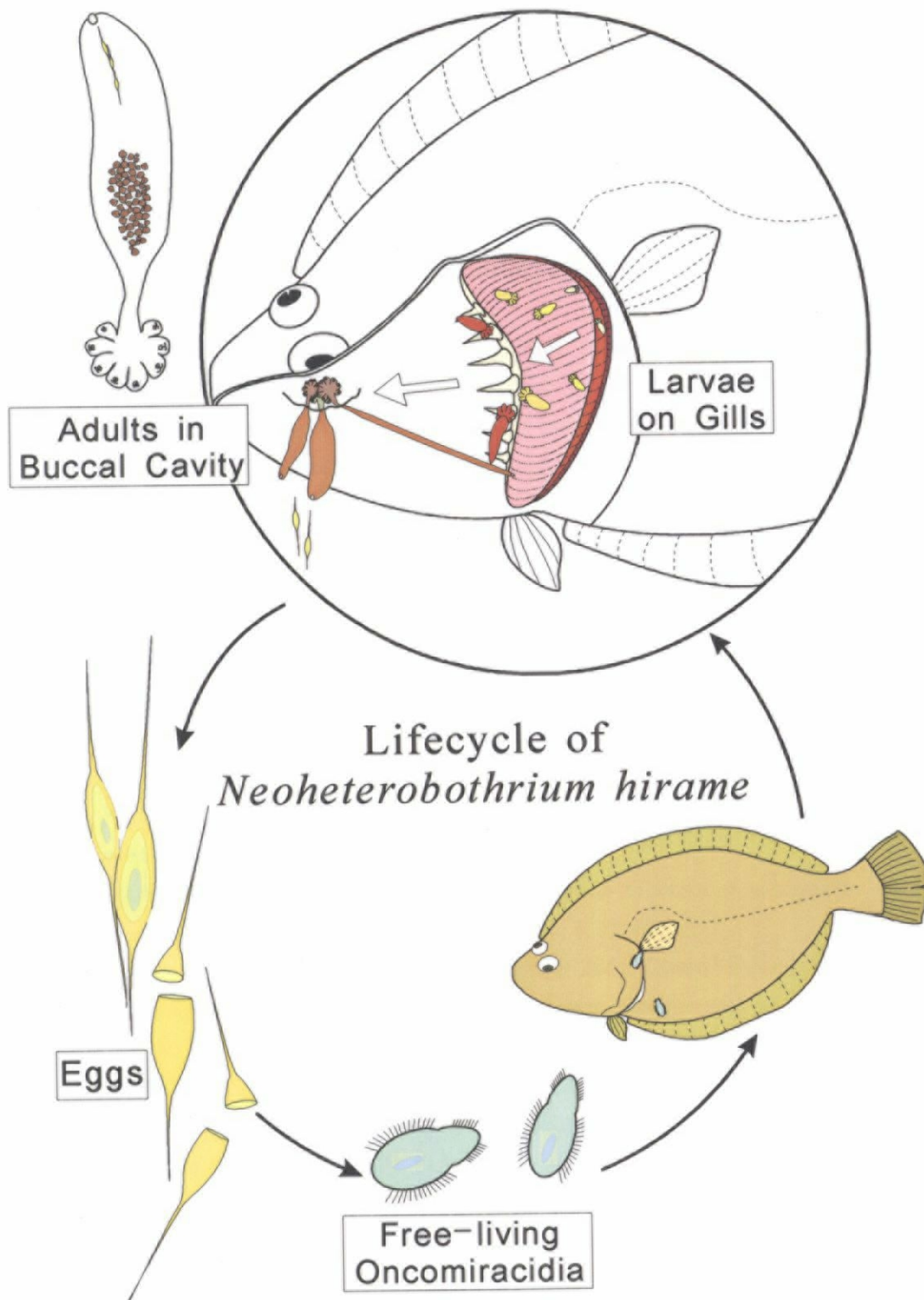


Figure I-4. Lifecycle of *Neoheterobothrium hirame*.

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Chapter II. Infection dynamics of *Neoheterobothrium hirame*:

Long-term monitoring and comparison of Obama and Miyako Bay.

INTRODUCTION

A recent epidemic of *N. hirame* infection is causing a rising concern for Japanese flounder fisheries. Past field studies have revealed considerable occurrence of anemic flounder and high prevalence of *N. hirame* in various Japanese waters (Yoshinaga *et al.*, 2000a; Mushiake *et al.*, 2001; Anshary *et al.*, 2001, 2002). As the *N. hirame* epidemic and the decline of flounder catch occurred at around the same time, people are suspecting a causal link between the *N. hirame* infection and reduction in the catch of host fish. However, in spite of the wide distribution of the parasite, changes in host population, estimated from the annual commercial catch, are highly variable among the localities.

The figure II-1 illustrates the magnitude of reduction in flounder catch in various prefectures since 1993. It appears that the reduction of flounder is more evident along the western coast of the Sea of Japan where anaemic flounder was first reported. For instance, in Tottori prefecture, the annual catch was over 250 t in 1995 but reduced to only 34 t by 2000 (MAFF) (Fig. I-3). This is more than 85% reduction within 5 years. Similar reduction in flounder catch has also been observed along the coast of the Sea of Japan as well as southern Pacific regions (Fig. II-1). In contrast, recent flounder catch in northern Pacific regions and in the Seto Inland Sea have been relatively stable, or even on the rise. For example, flounder catch in Iwate prefecture has increased for the past several years (Fig. II-2a).

Whether the parasite abundance in those regions negatively correlates with flounder catch is unknown. Longitudinal monitoring study of *N. hirame* has only been conducted in the limited area on the Sea of Japan (Anshary *et al.*, 2001), and a direct comparison in the *N. hirame* infection level between different geographical regions has

never been performed. To determine the differences in infection dynamics in the regions differing in change of flounder catch, a 2-year field study was conducted in conjunction with National Center for Stock Enhancement, Fisheries Research Agency (NCSE, FRA).

MATERIALS and METHODS

Collection Sites

Two representative locations, one with a consistent flounder catch and another with significant reduction of catch, were selected for the comparison of the infection dynamics of *N. hirame*. Wild juvenile flounder were collected monthly from January 2002 to January 2004 in conjunction with Miyako and Obama stations of NCSE, FRA.

Miyako Bay (39.37°N 141.57°E), Iwate prefecture, is located along the ria coast of Northern Pacific ocean, and is rather small, 24.1 km², but deep (max depth = 76 m) and elongated bay (Fig. II-3). The water temperature at Miyako Bay is relatively low, with the minimum temperature can be as low as 2.8°C (Fig. II-4a). The annual flounder catch in Iwate Prefecture which includes Miyako Bay, has been stable and even shows increase in these years (Fig. II-2a).

Obama Bay (35.29°N 135.43°E), Fukui prefecture, is located south-western coast of Sea of Japan (Fig. II-3). The *N. hirame* epidemic was first reported in this region. The bay covers approximately 58.7 km² with maximum depth of 35 m. The western Sea of Japan facing the significant reduction of flounder catch and the catch in Fukui prefecture also shows sharp decline within the past 5 years (Fig. II-2b). The water temperature at Obama Bay is considerably higher than that at Miyako Bay and the temperature rarely falls below 10°C (Fig. II-4b).

Fish Collection

Different methods for collection of juvenile flounder were employed at two bays.

At Miyako Bay, the majority of samples were collected using a slide net (opening size approx. 200 X 30 cm). Net towing was conducted every month at the relatively shallow, inner part of the bay. Although occasional net towing has also been carried out at Obama Bay, the large proportion of Obama samples was obtained from local shrimp boats, which often catch the juvenile flounder as a by-catch.

Collected fish were immediately fixed in 90% ethanol on the boat, or as soon as brought into the laboratory. After placing them in the fixative for at least several days, each fish was labelled with a serial number and following measurements and observations were carried out; total length (TL), standard length (SL), body weight, (BW), age (based on body size and/or otolith), and pigmentation of the blind side to distinguish wild fish from released seedlings. Based on the measurements, condition factor (K) was calculated for each fish using the equation $K = 100 \text{ BW} / \text{SL}^3$ where BW is in g and SL is in mm.

Parasite Count

Infection status of *N. hirame* in each collected fish was determined by following procedures. The operculum from eyed side was removed to expose buccal cavity and to safely excise the entire gill from the head. The excised gills were stored individually in a plastic bag in 95% ethanol. Once gills were removed, numbers of adult parasite inhabiting in the buccal cavity were counted under the dissecting scope.

Counting for the immature worms on the gills was carried out using the “stirring method” developed by Anshery *et al.* (2001) with minor modifications. Briefly, gills from each fish were cut into the small pieces and placed in a 500 ml beaker with 300 ml of tap water. Parasites were dislodged from the gill by stirring the water for 25 minute at approximately 1200 rpm. Large gill particles were removed by a strainer net (1 X 1 mm) and the suspension was filtered further with a micromesh net (20 µm) to trap all the worms. The trapped matters were observed under the dissecting scope

and worms were counted. To determine the developmental stages of each worm, all immature worms were mounted on a glass slide with glycerol jelly and light microscopy observation was performed. Individual worm was classified into one of the following five developmental stages based on the numbers of clamps; stage 0: no obvious clamps, stage I-IV; 1-4 pairs of clamps, respectively (Anshary *et al.*, 2001; Tsutsumi *et al.*, 2003). All worms found in the buccal cavity were classified as mature adult.

Statistical Analyses

To follow the infection dynamics of *N. hirame* on juvenile flounder over time, fish from 2001 (01), 2002 (02), and 2003 (03) year class (yc) were analysed separately. Samples with severe damage and/or unknown age class were excluded from the analyses. Differences in prevalence between the sampling location and the year class were analysed using Chi-square tests. For comparison of the parasite densities, a nonparametric Kruskal-Wallis test was carried out. A parametric t-test was used for the analyses involving fish size.

RESULTS

A total of 1,936 flounder, 977 from Miyako and 959 from Obama Bay, were examined during the study. Table 1 summarises the number of monthly samples based on the year class, location of capture (Miyako or Obama), and the origin (i.e. whether fish were wild or released seedlings). Numbers of monthly samples were highly variable among the months and only a few or no samples could be obtained in some months. Changes in the fish size for each year class were plotted on Fig. II-5. In general, for the fish captured in the same month, those from Obama were larger than those from Miyako Bay (t-test $p < 0.01$).

Miyako Bay

A total of 153, 312 and 512 fish was examined from the 01, 02 and 03 yc, respectively. Fig. II-6 illustrates the seasonal dynamics in the *N. hirame* infection in each year class. In general, *N. hirame* infection tended to be low during the summer and increases in the autumn remains high during throughout winter and the early spring of the following year. The significant fluctuation in infection prevalence has been observed. There was a rapid increase in the prevalence during August-December. The timing of rise in infection prevalence followed the settlement period of the juvenile flounder and corresponded with fall of water temperature. Prevalence remained high during the winter and then gradually declined by the following spring and became low between April-July as seen in 02 yc. The highest prevalence observed for the ycs 01 (in April and October), 02 (in December) and 03 (in December) were 100, 92 and 56 %, respectively. However, it has to be noted that the value for the 01 yc was based on small sample numbers.

After facing the primary infections as young-of-the-years (yoy), fish were challenged by a second major infection as 1-year old. The prevalence for the 01 and 02 yc showed the sharp second increase in the September of the second year. It is apparent that the initial increment of *N. hirame* infection for yoy occurred shortly after the increase of infection prevalence in the previous generation (1-year old).

Although a precise comparison among the three yc is difficult due to the sporadic samples, overall infection seemed to be on decline over the study period. The annual prevalence for the 01, 02 and 03 ycs were 51.6, 23.7 and 14.1%, respectively, and were statistically different from each other ($\chi^2=85.81$, $P<0.001$). However, it has to be noted these values were based on the limited sample size and samples were lacking in some months.

Unlike prevalence, the mean intensity (i.e. numbers of worms per infected fish) was relatively stable throughout the year (Fig. II-6). The monthly mean intensity never

exceeded 4 worms, except in October for 01 yc, which represents only one fish. There is also a declining tendency of mean intensity over three generations. The annual mean intensities (\pm SD here and elsewhere in paper unless otherwise stated) for the 01, 02 and 03 ycs were 2.59 ± 1.94 , 1.82 ± 1.4 , 1.50 ± 0.87 , respectively, and were significantly different from each other (Kruskal-Wallis $P < 0.001$).

No apparent difference in *N. hirame* infection between wild and released flounder was observed (Fig. II-7). Released fish showed similar infection dynamics, in both the prevalence and the intensity, to the wild fish. Although the infection level seemed to be higher in wild fish for 03 yc, a detailed comparison was not performed due to small sample sizes.

The analysis for the parasite's developmental stage demonstrated the clear infection cycles (Fig. II-8). For example, high proportions of primary stage larvae were observed in yoys during September-December for the 02 yc (Fig. II-8b). Only a minute proportion of adult worms were found in September, but their number increased through October and November. Numbers of early development staged immature worms re-increased in December. This second increase of immature worm was corresponded with the rapid increase of parasite abundance. As seen from the 01 yc, immature parasite re-appeared every 2-3 months. This suggests that the span of *N. hirame* generation change during the summer period is 2-3 months (Fig. II-8a).

Although the study was aimed for *N. hirame* infection, considerable numbers of adult digenean trematodes were found from wild flounder. There were at least two species of trematode and one was identified as *Uteroverisculus* sp., probably *U. paralichthydis* which inhabits in the stomach of olive flounder (Ohtuka *et al.*, 1980). Other remained unidentified. Although these worms were found from the gills along with immature *N. hirame*, it is unlikely that gills are the infection site for the trematode. One possibility is that these digenean worms migrated upward to the esophagus and had been vomited out by the host during the fixation process. Overall

prevalences for the trematode were 19.0, 10.9 and 13.7 % for the 01, 02 and 03 yc, respectively. The mean intensities ranged from 2.2 to 3.5 with the highest value of 17 worms per fish. However, these numbers are probably lower than the actual infection levels as many worms may still be remained in their original infection site. Trematodes were most abundant between July-September.

Obama Bay

At Obama Bay, the 01 yc samples were obtained only from 4 sporadic months. Therefore, the analyses of infection dynamic were primarily based on the 02 yc with additional data from the 03 yc. Infection dynamics of *N. hirame* at Obama Bay were basically similar from that at Miyako Bay. The infection level increased in autumn to winter followed by reduction in parasite abundance during the summer (Fig. II-6b).

The rapid increase in prevalence started in August and reached 100% by December-February. Corresponding to the increase in prevalence, the mean intensity also rose significantly. For the 02 yc, the difference between the highest and the lowest worm intensities was nearly 20 fold. The annual mean intensities for the 01, 02 and 03 yc were 6.67 ± 12.09 , 5.35 ± 6.8 and 3.76 ± 3.31 worms, respectively, and there was no statistical differences between years (Kruskall-Wallis $p=0.51$). The highest observed intensities for the 01, 02 and 03 yc were 47, 42 and 22 worms per fish, respectively.

A comparison between wild and released fish was carried out only for the 02 and 03 yc as all 01 yc samples were released seedlings (Fig. II-9). The infection dynamics between wild and released fish were similar. The only noticeable, but slight, difference was that the prevalence of the released fish reached to the maximum value in somewhat faster rate. No significant difference in the overall parasite intensity between the two groups were observed for any year classes (Wilcoxon, $p>0.05$).

A detailed analysis of infection cycle was difficult for Obama Bay as the majority of

larvae were at stage III or later (Fig. II-10). Immature worms were observed throughout the year, but their proportions tended to be higher in winter, when the infection level was on the rise. For the 02 yc, stage I and II worms were observed only in December and February. Interestingly, some worms found in the gills contained eggs. To my best knowledge, this was the first report of gravid *N. hirame* from the gills. They tended to be larger in size compared to regular immature ones. This gravid gill worm was unique to Obama samples and was not observed from Miyako. Gravid gill worms were found only from the 03 yc, and most were on the fish captured in January, 2004. Trematodes, similar to ones found in Miyako, were also found from the fish captured in Obama Bay, but total number of worm was less than 10.

A significant negative correlation between worm intensity and hosts' condition factor (K) was observed from the 02 yc ($r=-0.23$, $p<0.0001$, $N=633$) (Fig. II-11). The condition factor decreased during winter, and it was corresponded with high parasite abundance (Fig. II-12). Fish condition recovered in summer when the infection level lowered. Similar relationship between condition factor and infection was also observed on the 01 yc, but no further analysis was performed because of the small sample size.

Miyako vs Obama Bay

The prevalences and the mean intensities for each yearclass for both Miyako and Obama Bay were plotted on the same graphs (Fig II-13). The overall intensity in Obama Bay was significantly greater than that from Miyako Bay, except for the 01 yc (Wilcoxon rank sum test; $Z = -6.59$ for 02 yc, $Z = -7.09$ for 03 yc, $p < 0.001$). Worm intensities were mostly below 4 worms per fish at Miyako while those often exceeded more than 10 at Obama. The highest observed intensity from Miyako and Obama Bays were 10 (01 yc in January, 2002) and 47 (01 yc in March, 2003), respectively.

The maximum prevalence exceeded 90% in both bays. The first infected yoy were captured in May at Obama while infection was not observed until September at Miyako. Corresponding to this earlier establishment of infection, the prevalence at Obama reached to the maximum value a few months earlier than that at Miyako Bay.

DISCUSSION

This two-year field study revealed the striking difference in the infection intensity of *N. hirame* between Miyako and Obama Bay while the prevalence remained comparatively similar. The annual mean intensity for 02 yc at Obama Bay, where the flounder catch showed significant decline, was almost three fold higher than that at Miyako, an area with increasing catch. Although to determine the precise effects of *N. hirame* on the wild juvenile flounder requires further investigations, accordance between the parasite intensity and change in flounder catch at these two localities is suggestive. Anshary *et al.* (2002) suggested that *N. hirame* caused mortality of 0-year-old flounder based on their field observation at Wakasa Bay. Moreover, the positive correlation between the severity of anemia and the adult *N. hirame* intensity (Mushiake *et al.*, 2001) has been reported. Given the pathogenicity of *N. hirame* is intensity-dependent, higher worm intensity observed at Obama Bay suggests the greater effect of *N. hirame* on flounder population at Obama than that at Miyako Bay.

Various factors could have caused the observed difference of the parasite intensities between two areas (reviewed in Bush *et al.*, 2001). Both biotic and abiotic factors contribute to the various aspects of the parasite life history and caused spatial difference in *N. hirame* abundance. Possible factors includes chemical composition of the water, water temperature, physical characteristics of the bay, climatic conditions, host density, host genetics, host ecology, and/or intra- and interspecific competitions (e.g. Marcogliese & Cone, 1991; Hartvigsen & Halvorsen, 1994; Bagge, 2004). However, specific effects of above factors on *N. hirame* are poorly known. Moreover,

detailed information on these parameters from the two study areas is limited. Considering these, identifying the underlying mechanisms causing the differences in parasite intensity at Obama and Miyako Bay is a difficult task.

It seems a general tendency that the reductions of flounder catch are more prominent in the Sea of Japan side than the Pacific side, and more apparent in the southern (western) than the northern area. Between 1993 and 2002, more than 80% reductions in flounder catch have been observed in Tottori, and the Sea of Japan side of Hyogo prefecture (Fig. II-1). High reduction of flounder has also been observed around Wakasa Bay, Pacific side of Kyusyu, and Southern Shikoku. The water temperatures in these regions are usually high and rarely falls below 10°C. In contrast, the flounder catch has been relatively stable on the northern Pacific coast, Kanto regions and in the Seto Inland Sea. The water temperature in the northern Pacific regions such as in Iwate, is extremely low (Fig. II-4a). Although the Seto Inland Sea is located in the southern part of Japan, water temperature falls below 10°C during the winter months (Japan Oceanographic Data Center). Such differences in the water temperature may play an important role in the spatial variation in *N. hirame* abundance, thus the reduction of the host populations.

At both Miyako and Obama Bay, two major peaks of infection were consistently observed. The initial increase of infection occurred in Sept.-Dec. at Miyako, and June-Dec. at Obama Bay. This rapid parasite recruitment indicates the high parasite transmission among the naïve yoy flounder. Hi transmission rate is probably facilitated by high density of juvenile flounder in the nursery ground. At Miyako, first infection was confirmed in Sept. from the fish with average SL of approx. 65 mm. Although, majority of parasites at that time was immature larvae, some fish already bore adult worms. In contrast, first infection was observed as early as in June at Obama from the fish of approximately 90 mm in TL. Considering *N. hirame* takes about 40 days to develop into the adults at 20°C (Tsutsumi *et al.*, 2003), the first

infection to yoy flounder may have occurred around August at Miyako or by May at Obama Bay. Breeding season of flounder in Wakasa Bay is between March and April while that in Iwate delays for several months, mainly in June-July (Minami, 1997). Flounder takes 1-1.5 month after hatch until they complete metamorphosis and to be settled (Tanaka, 1997). In a simple calculation, flounder at Obama Bay are exposed to *N. hirame* several months longer compared to that at Miyako Bay. Such longer exposure time may contribute to the higher parasite intensity at Obama.

Numbers of infection cycles (numbers of parasite generations appears in specific time period) between the initial infection and the time at peak infection are at least 2-3 times in Miyako and probably 5-6 times in Obama. *N. hirame* takes 72, 42, and 30 days at 15, 20 and 25°C, respectively, to reach maturity from a newly laid egg (Yoshinaga *et al.*, 2000b; Tsutsumi *et al.*, 2003). At Miyako, first adults appeared in Sept. and their progeny appeared as Stage-I larvae in Oct. and Nov. Large proportions of early-stage larvae found in Dec. are probably the third generations. On the other hand, no stages I-II larvae were found at Obama except in Dec. and Feb. The average water temperature in Obama between June and Dec. is approximately 22°C, thus infection cycle from the first appearance to the infection peak can be estimated as approximately 6 times.

Interpretation of the decline in infection level after the peak infection in Dec-Feb needs some careful thoughts. Possible explanations include reduced transmission rate, mortality of infected host, mortality of parasite, and/or reduced capturing rate of infected fish. Host density and host population size have both been shown to be a determinate factor for the parasite abundance and parasite community (Arneberg *et al.*, 1998; Morand & Poulin, 1998, Bagge *et al.*, 2004). Juvenile flounder leave nursery ground to migrate toward the deeper waters as they shift food preference and become piscivorous (Noichi, 1997). Small sample size during the spring period may also indicate the habitat change of flounder. Migration and dispersion cause a reduction in

host density and may reduce the contact chance between the parasite and the host (McCallum *et al.*, 2001; Bagge *et al.*, 2004). As adult *N. hirame* eventually detach from the host (Anshary and Ogawa, 2001), and if new recruitment is limited due to low transmission rate, parasite population is expected to shrink. In addition, *N. hirame* may be incapable to infect or have reduced infectivity, and may have lower survival rate under the low temperature condition.

Decline of *N. hirame* infection level can be explained solely by the mortality of infected host (Anshary *et al.*, 2002). The significant and negative correlation between the parasite intensity and host's condition factor also seems to support this. Furuta (1998), in both laboratory experiments and field studies, showed that mortality of juvenile flounder due to predation and cannibalism increase when food resources are depleted. Thus, it is conceivable that the parasite causes reduction in host condition factor and subsequently increases the mortality. However, it is also highly possible that the fluctuation of host's condition factor and the parasite intensity is an independent phenomenon. Depleted food resources during winter cause reduction of fish body weight, and it coincidentally accord with increase of parasite intensity that happened to occur during the same time. No study has yet to demonstrate the direct effect of *N. hirame* on host's mortality. Therefore, further studies are necessary before the clear link between *N. hirame* and mortality of wild juvenile flounder can be established.

Low infection levels during spring-summer months are probably the result of a lower transmission rate owing to low host density. However, infection level shows a second increase between July-August of the following year. Dispersed flounder may have returned to the shallower water during this period. This is supported by the increase of the capturing rate of 1 year-old flounder during this period. One possible reason for the reappearance of 1 year-old is to feed newly settling flounder larvae. Cannibalism in flounder between and among the year class has been well documented.

In fact, 1-2 years old flounder are shown to be one of the main predators of juveniles (Yamashita, 1997). Gathering of 1-year old flounder probably facilitate transmission of *N. hirame* among the population of 1-years and also to the 0-years. The infected 1-years of older flounder spread the parasite eggs to the nursery ground and the parasite may rapidly spread to the naïve yoy. As older flounder and yoy have been captured from the same sampling, it is certain that they share the same habitat, at least in some time of the year.

Stocking of seedlings may also facilitate parasite transmission. For instance, in 2002, 1,204,000 and 329,000 juvenile flounder were released in Iwate and Fukui prefectures, respectively. The present study showed that released fish accumulated *N. hirame* at a very fast rate and become comparable to the wild fish within a few months. Mass releasing of naïve hosts temporarily increase the density of susceptible host and resulting in increase of the parasite transmission rate among both released and wild fish. In 2003, Tottori prefecture terminated the stocking program as high stocking efficiency cannot be anticipated. Comparison of parasite abundance prior to and after the stocking termination will provide useful information. Releasing of juvenile flounder is still widely practiced in other regions. To minimize the parasite transmission, timing, location, and stocking size should be carefully planned.

In summary, the present study demonstrated the possible link between the population decline of olive flounder and the intensity, but not prevalence, of *N. hirame*. However, the study is limited on the two localities and it would be better not to come to a hasty conclusion. To determine the effects of *N. hirame* on host survival and to assess whether the effect is intensity-dependent, experimental studies are mandatory. *N. hirame*-flounder can be a model system to investigate the effect of introduced parasite on the host population, and continuous monitoring of this parasite in more locations will provide further evidence and valuable information on this matter.

Table 1. Numbers of wild (W) and released (R) fish captured in each month at Miyako and Obama Bay during January 2002 to January 2003.

Sampling time		2002												2003												2004	
		Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Jan	
MIYAKO BAY	2001	W	31	4	3				21	32	9	4	25	1													
		R	5	2	1	1			2	5	2		5														
	2002	W											70	17	12	3			2	14	2	1					
		R											96	36	20	10			4	2	13	7	3				
MIYAKO BAY	2003	W																		13	43	17	16	21			
		R																		0	152	116	45	40	25		
	Total		36	6	4	1	23	37	11	4	196	54	32	13	0	0	6	0	2	40	204	138	61	65	39	0	
OBAMA BAY	2001	W																									
		R								21	13					1	5										
	2002	W								60	68	57	39	41	80	28			31	19	1	2	0	1	0	1	
		R								2		7	12	24	26	11			11	21	1	2	1	1	0	1	
	2003	W																			34	65	29	34	0	18	
		R																			29	30	23	16	2	6	
Total		0	0	0	0	0	0	83	81	64	51	65	106	39		12	82	0	0	42	40	65	99	53	52	2	26

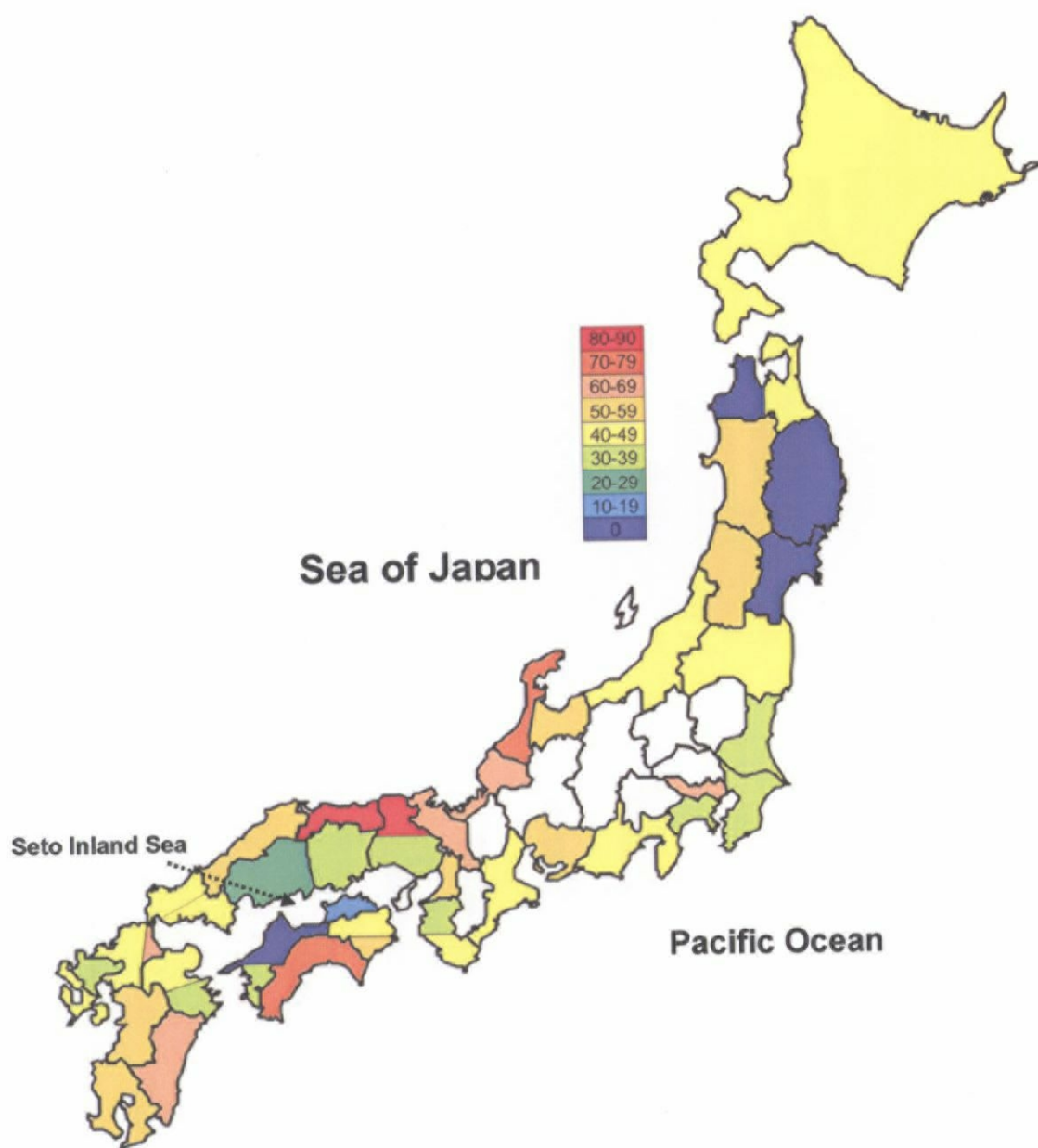


Figure II-1. Rate of reduction in annual flounder catch during 1993-2002 in each prefecture. The rate indicates the difference between the maximum annual catch and minimum catch recorded in subsequent years. Aomori, Hyogo, Wakayama, Yamaguchi, Ehime, Tokushima, Oita and Fukuoka were divided into 2 regions based on the locations of the seas (e.g. Coast of Aomori consists of both Pacific and the Sea of Japan). Values were calculated from the statistical data from The Ministry of Agriculture, Forestry and Fisheries of Japan.

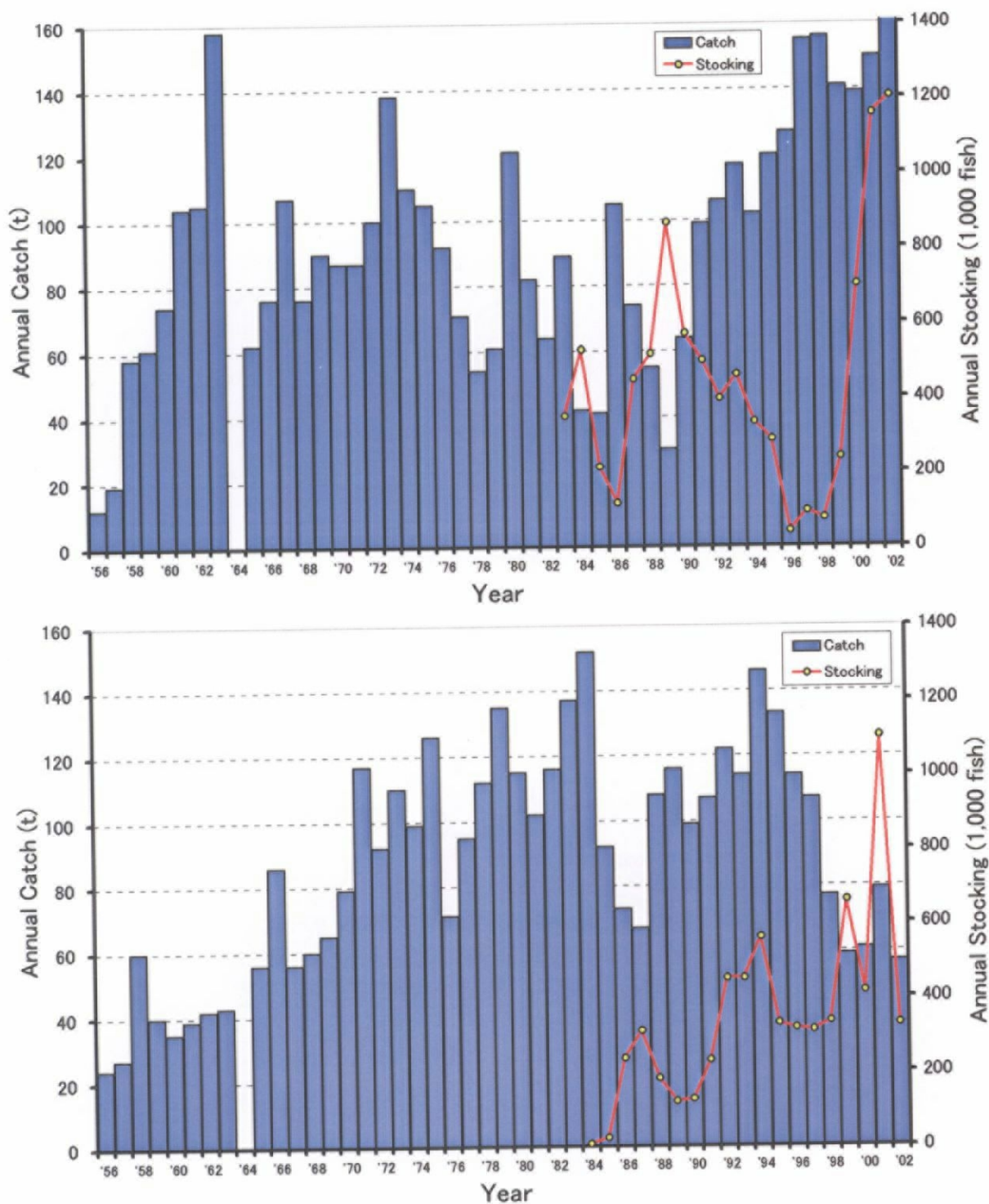


Figure II-2. Commercial annual catch and stocking of olive flounder in Iwate (a: top) and in Fukui (b: bottom). Notice the sharp decline in catch at Fukui starting mid-90s. Data obtained from Agriculture, forestry and fishery statistics (The ministry of agriculture, forestry and fisheries of Japan) and National Center for Stock Enhancement.

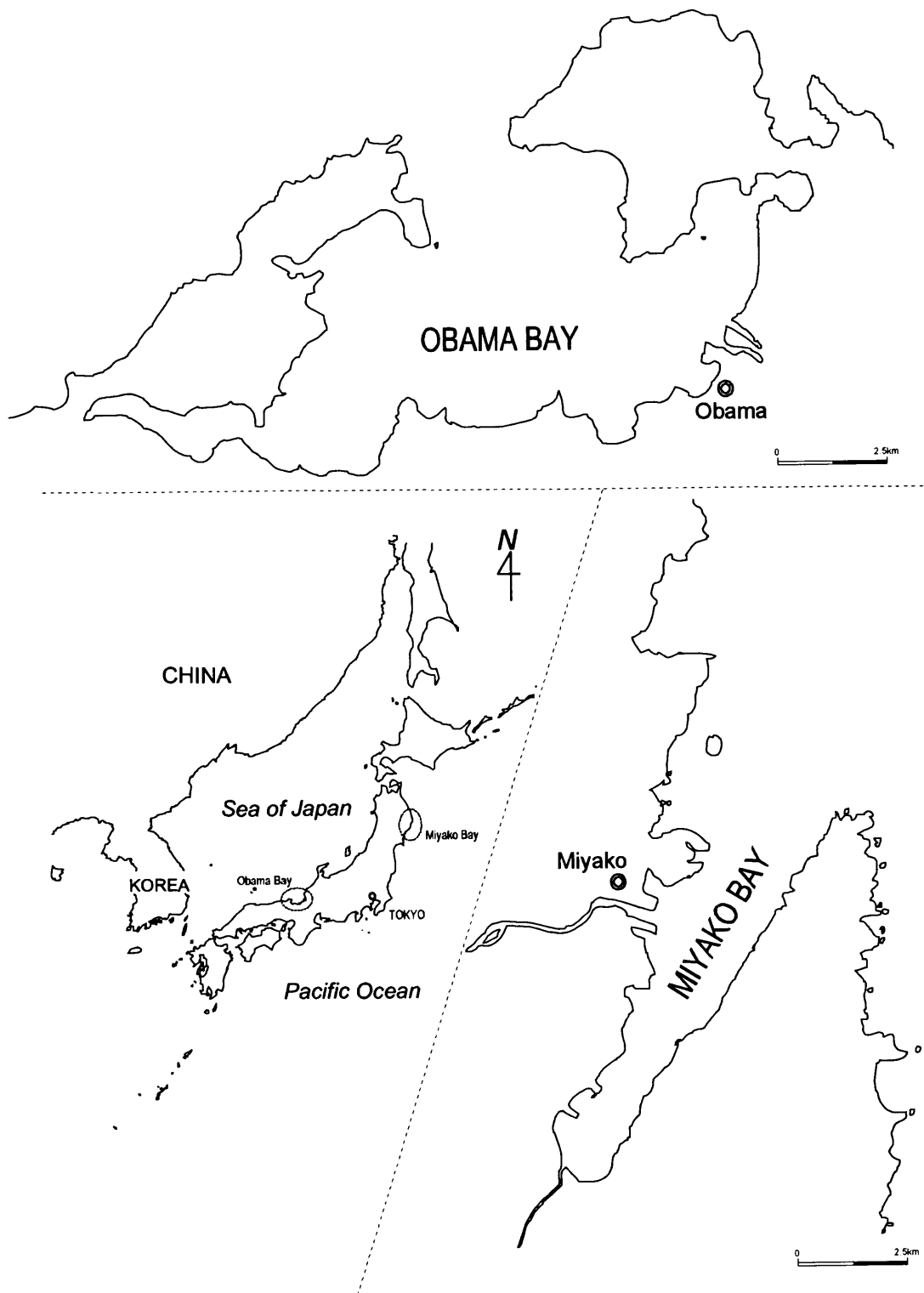


Figure II-3. Map of Obama bay, top and Miyako bay, bottom right.

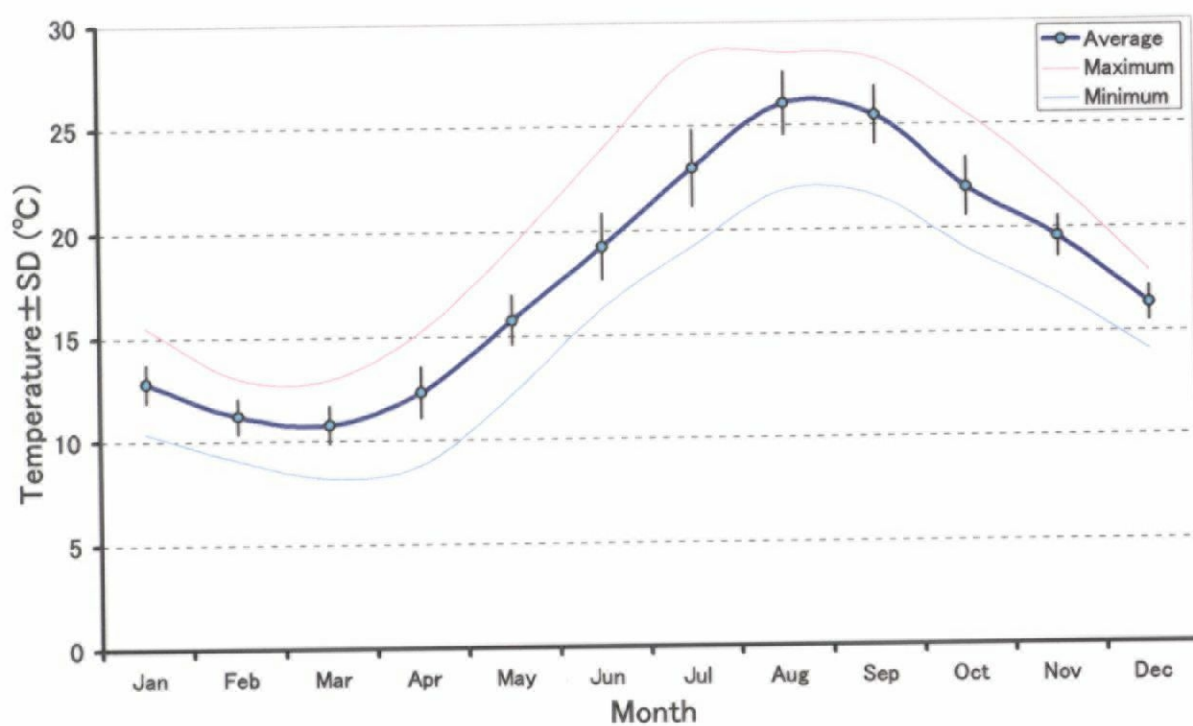
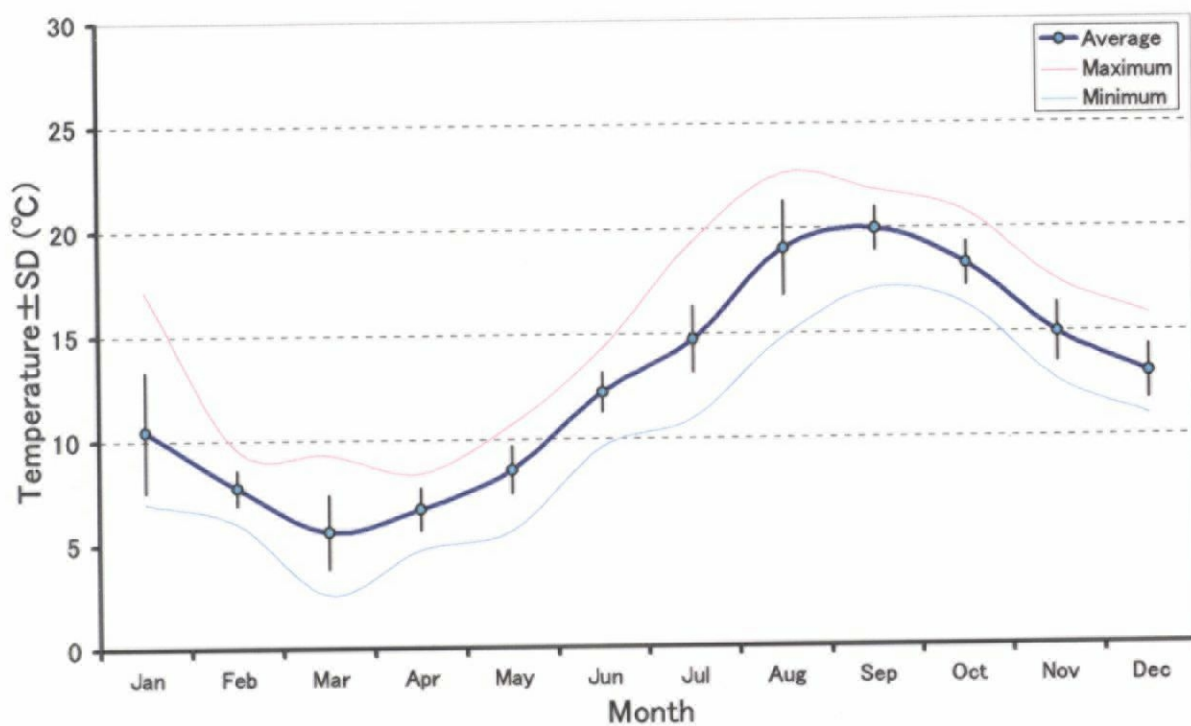


Figure II-4. Annual change of water temperature (monthly average, max. and min.) at 10m depth in Miyako bay area (a: top) and Obama bay area (b: bottom). Original data obtained from Japan Oceanographic Data Center (based on measurements from 1906 to 2003).

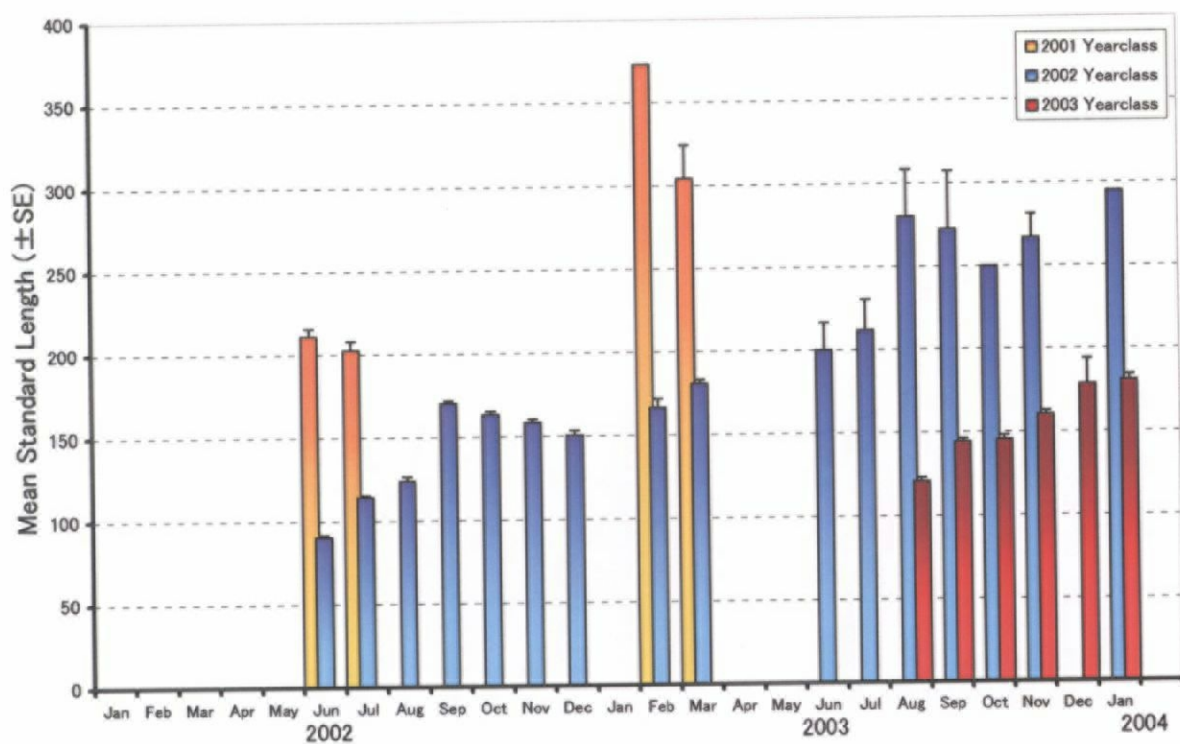
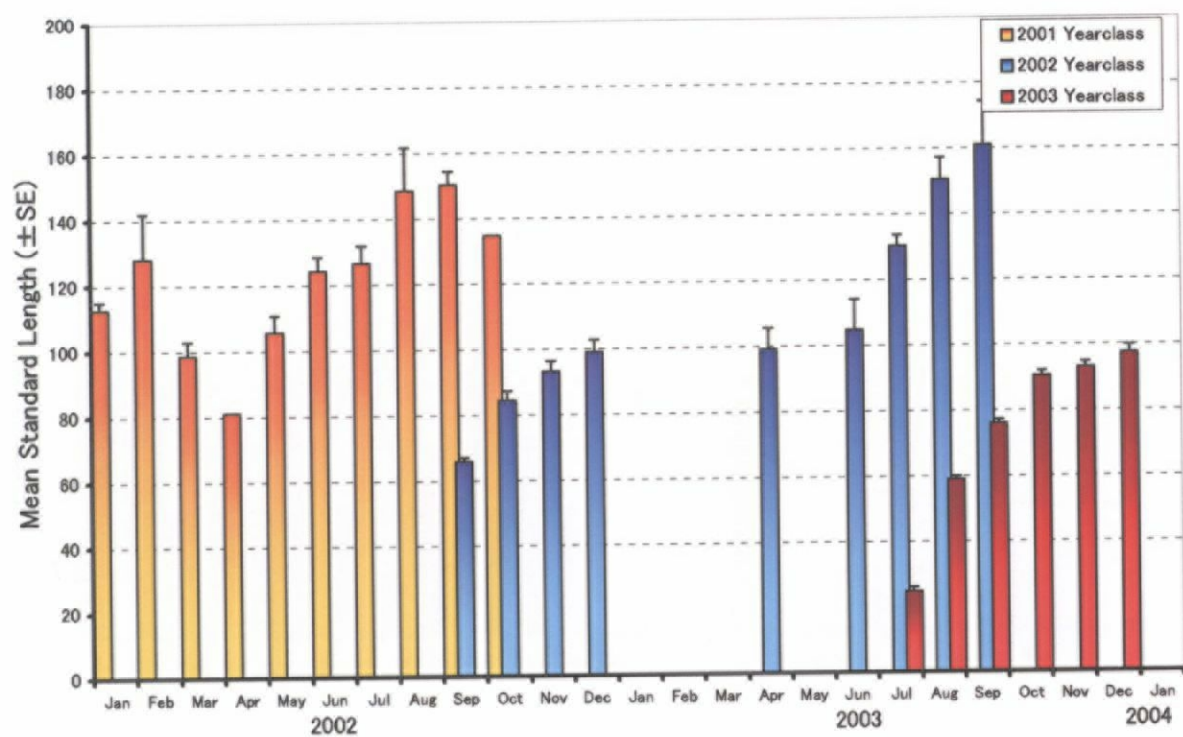


Figure II-5. Standard length of sample fish from 2001, 2002, and 2003 yearclass capture at Miyako (top) and Obama bay (bottom).

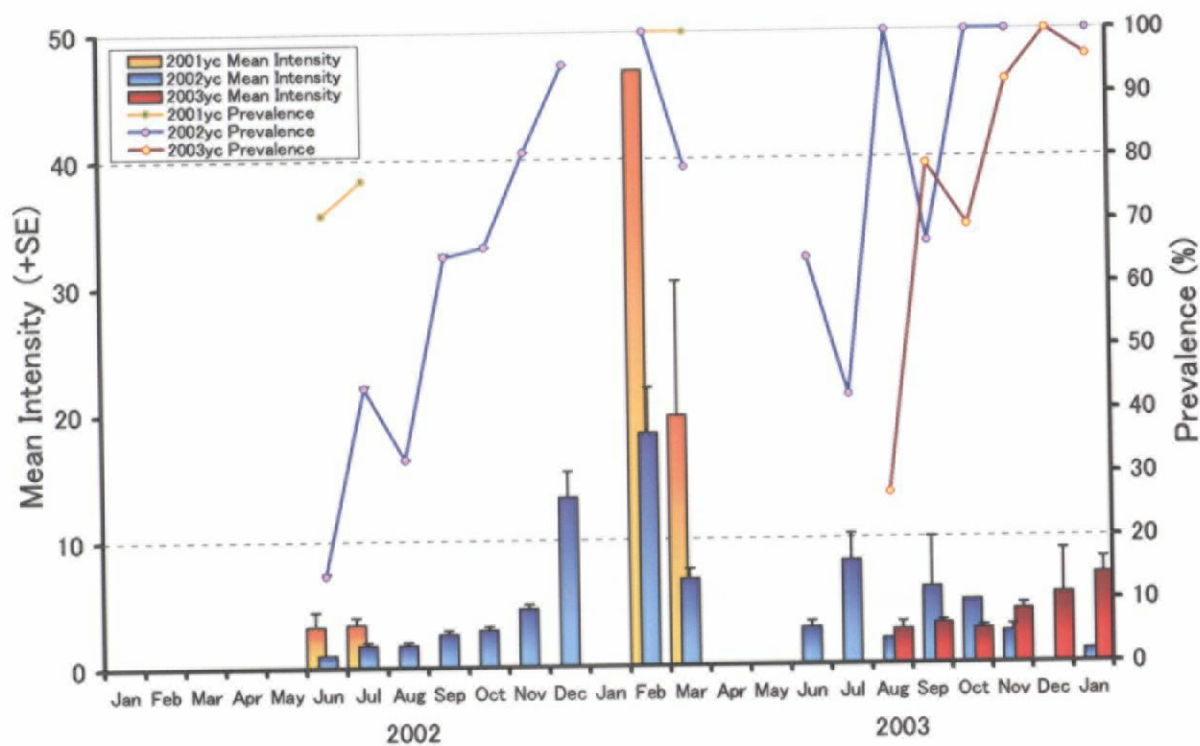
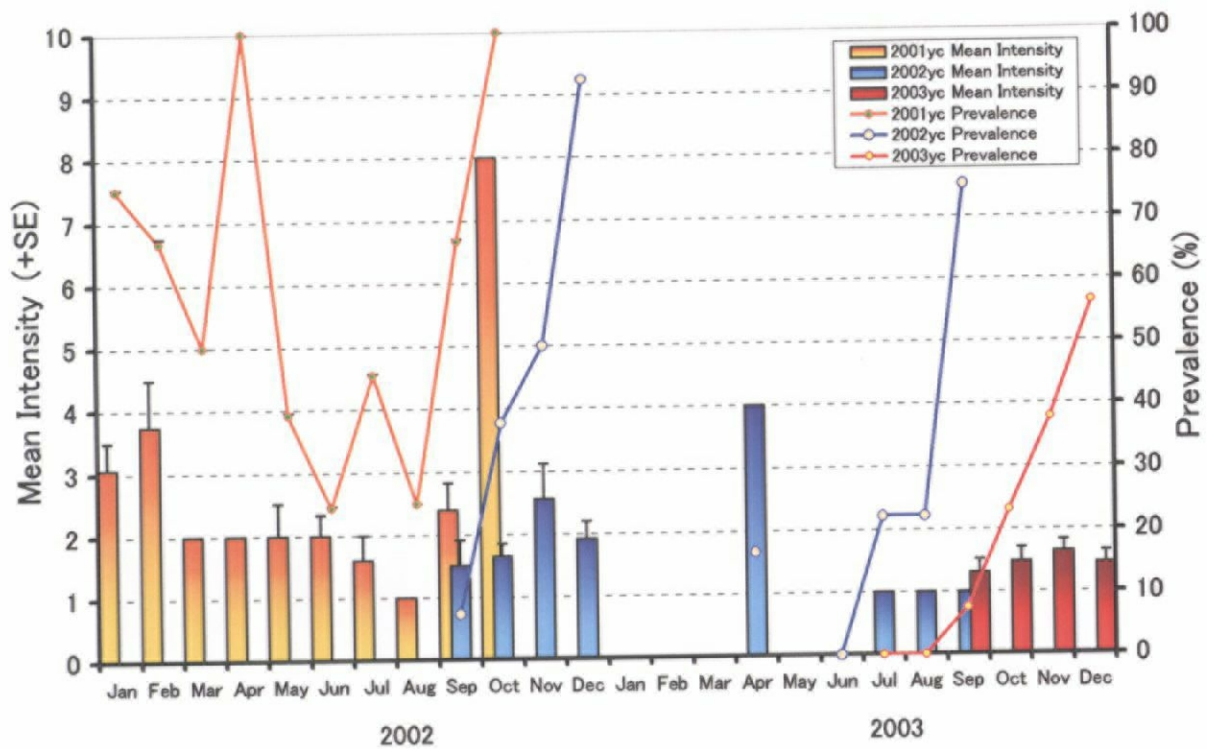


Figure II-6. Prevalence and mean intensity of *Neoheterobothrium hirame* in juvenile flounders of 2001-2003 yearclass from Miyako (a: top) and Obama (b: bottom)

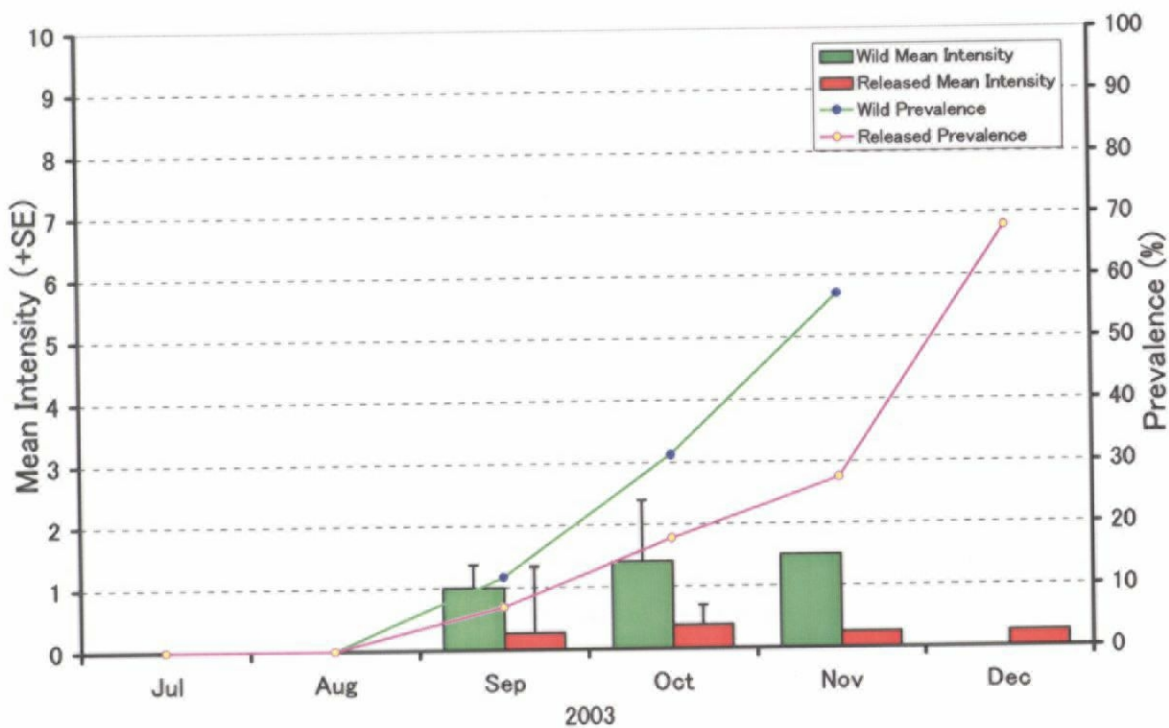
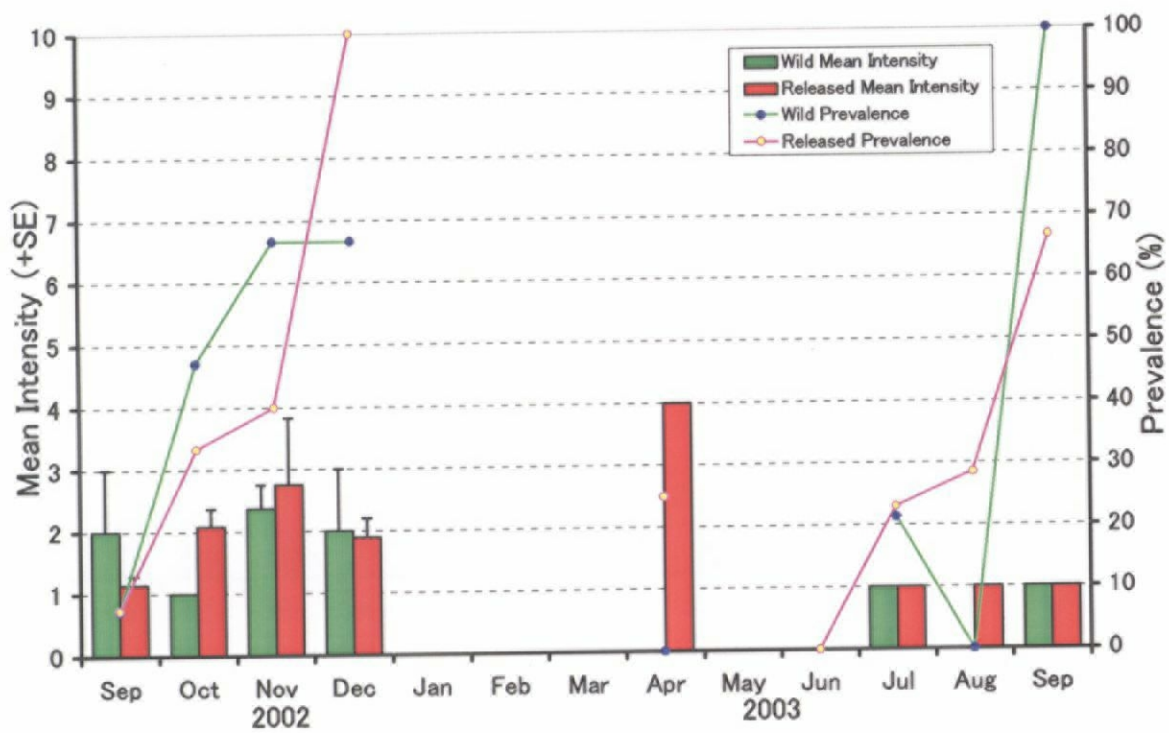


Figure II-7. Prevalence and mean intensity of *Neoheterobothrium hirame* in wild and released juvenile flounder of 2002 (top) 2003 (bottom) yearclass captured in Miyako bay

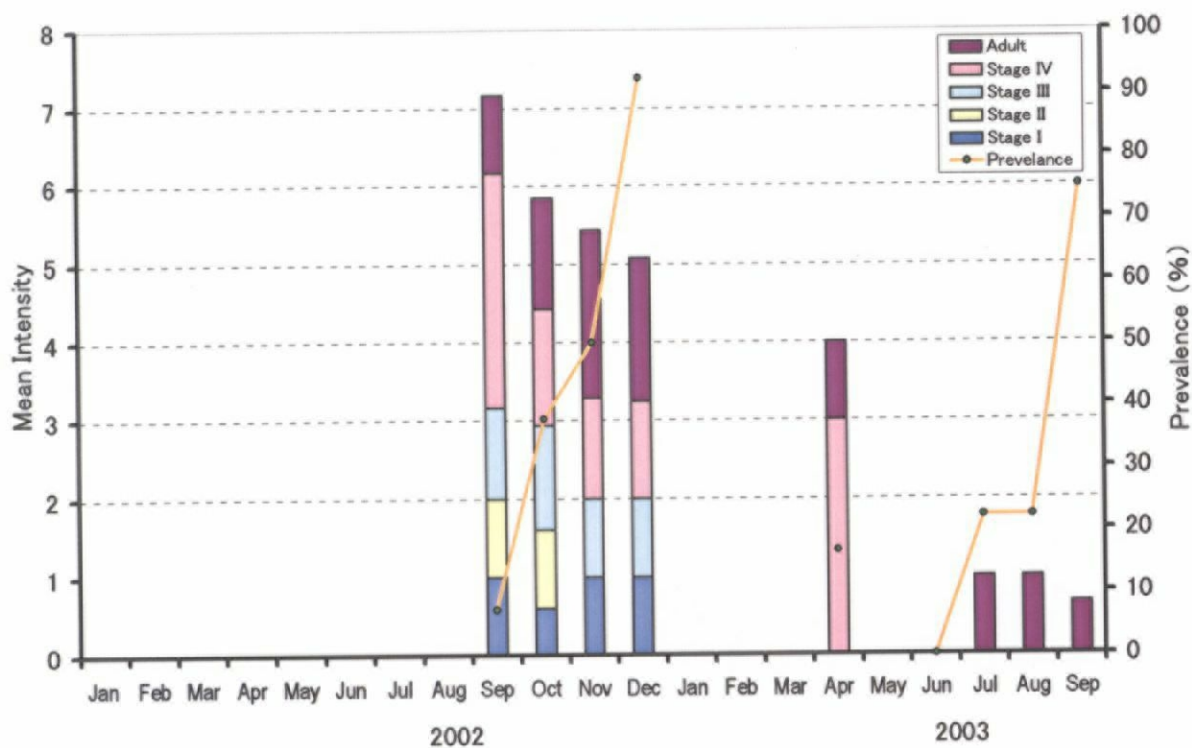
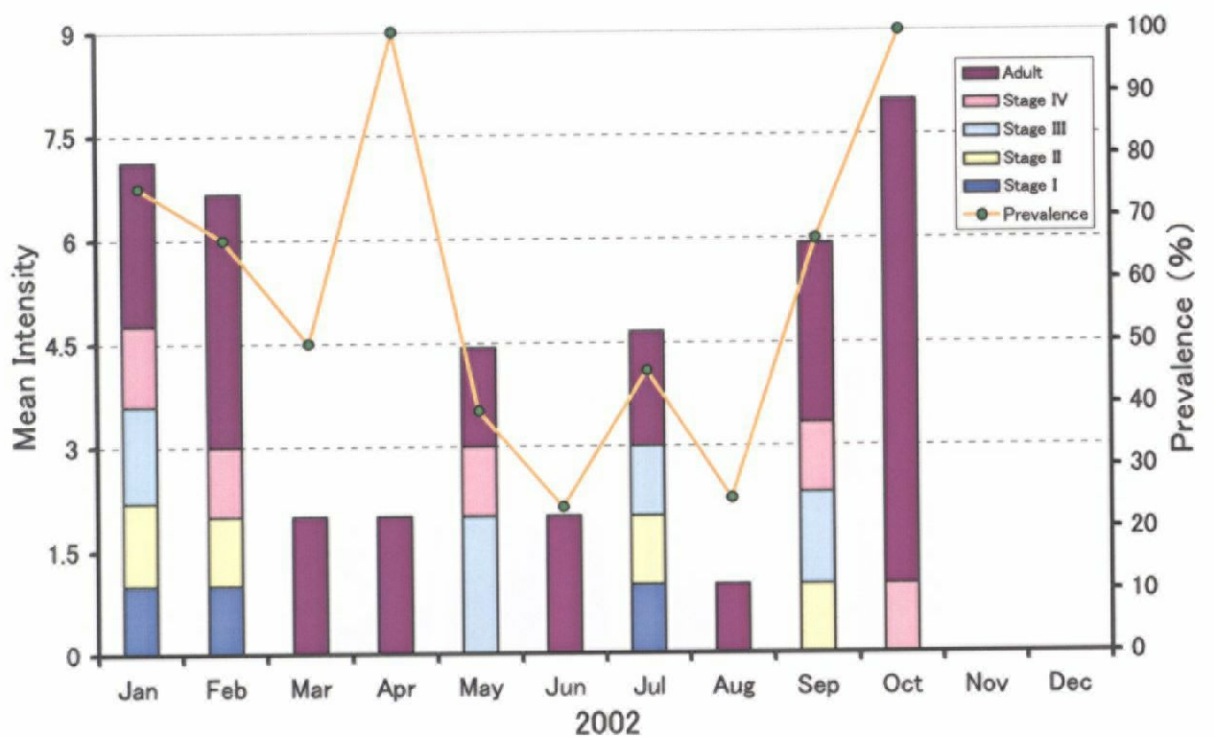


Figure II-8. Prevalence and mean intensity of various development stages of *Neoheterobothrium hirame* from 2001 yc (a: top) and 2002 yc (b: bottom) at Miyako Bay.

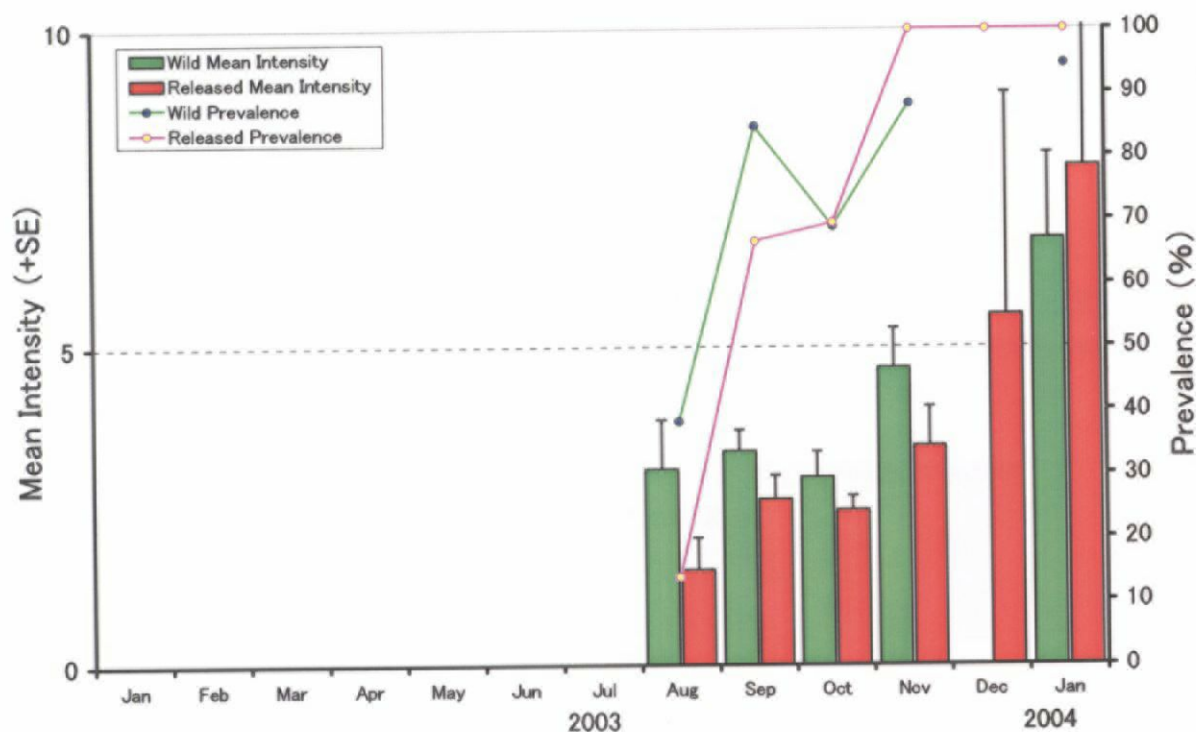
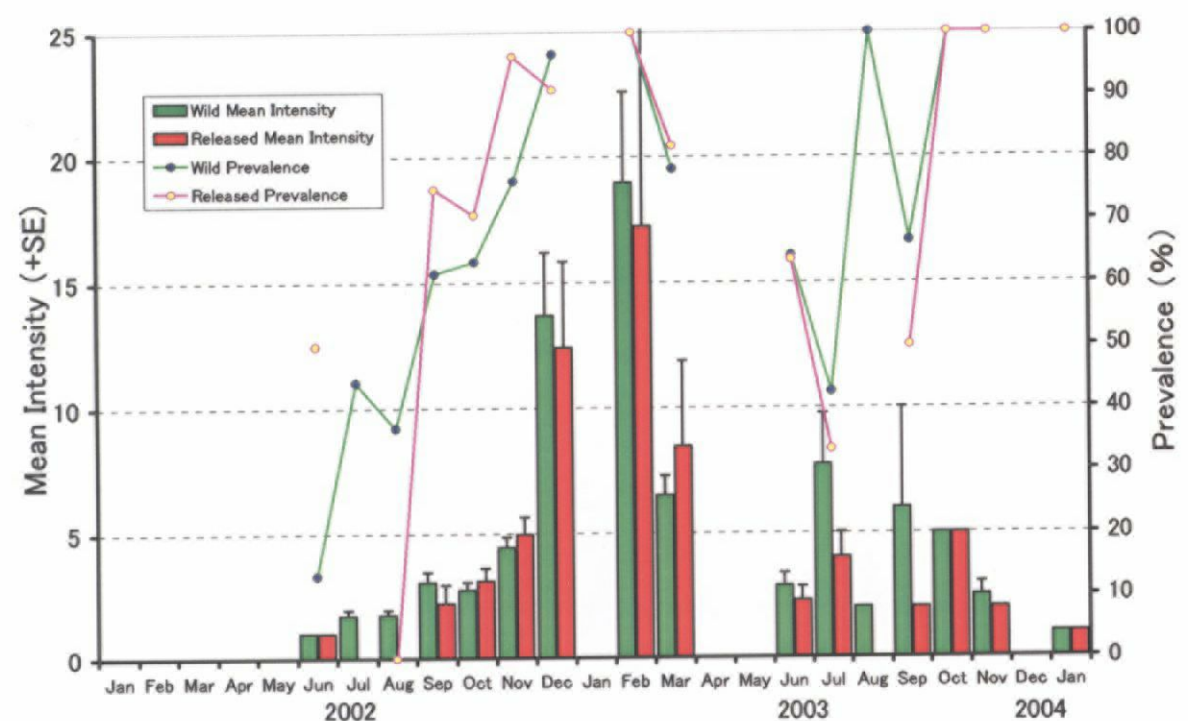


Figure II-9. Prevalence and mean intensity of *Neoheterobothrium hirame* in wild and released juvenile flounder of 2002 (top) 2003 (bottom) yearclass captured in Obama Bay

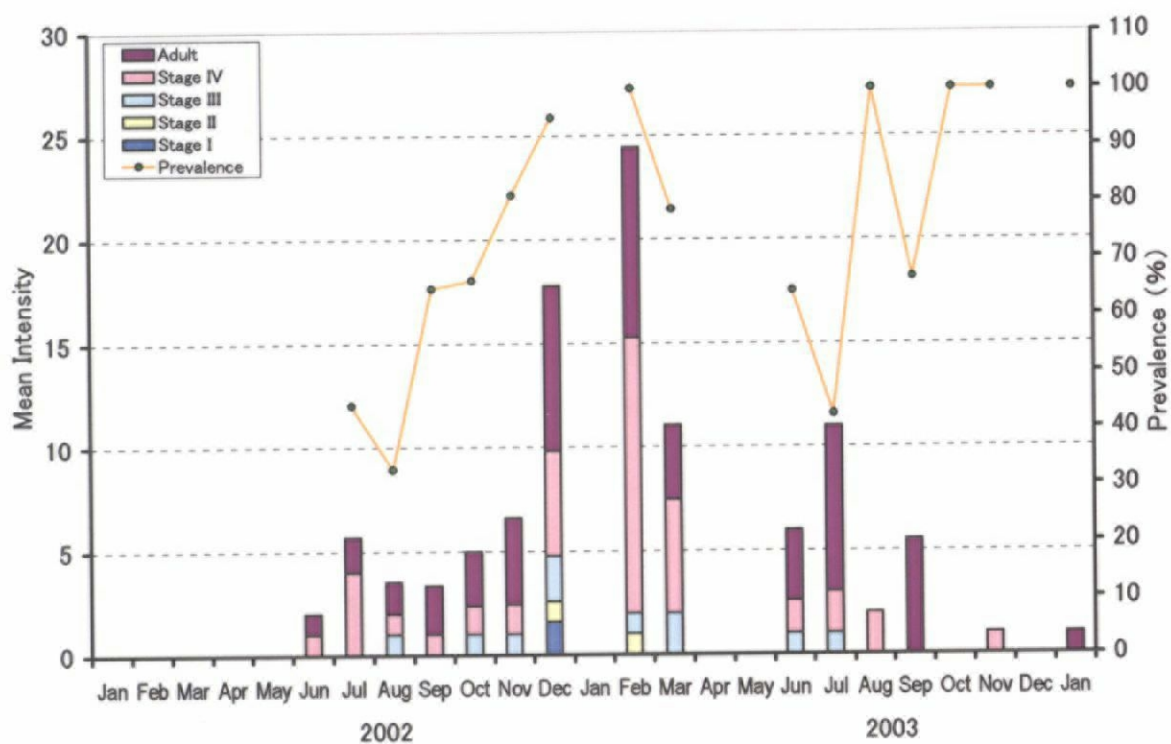


Figure II-10. Prevalence and mean intensity of various development stages of *Neoheterobothrium hirame* from 2002 yc at Obama Bay.

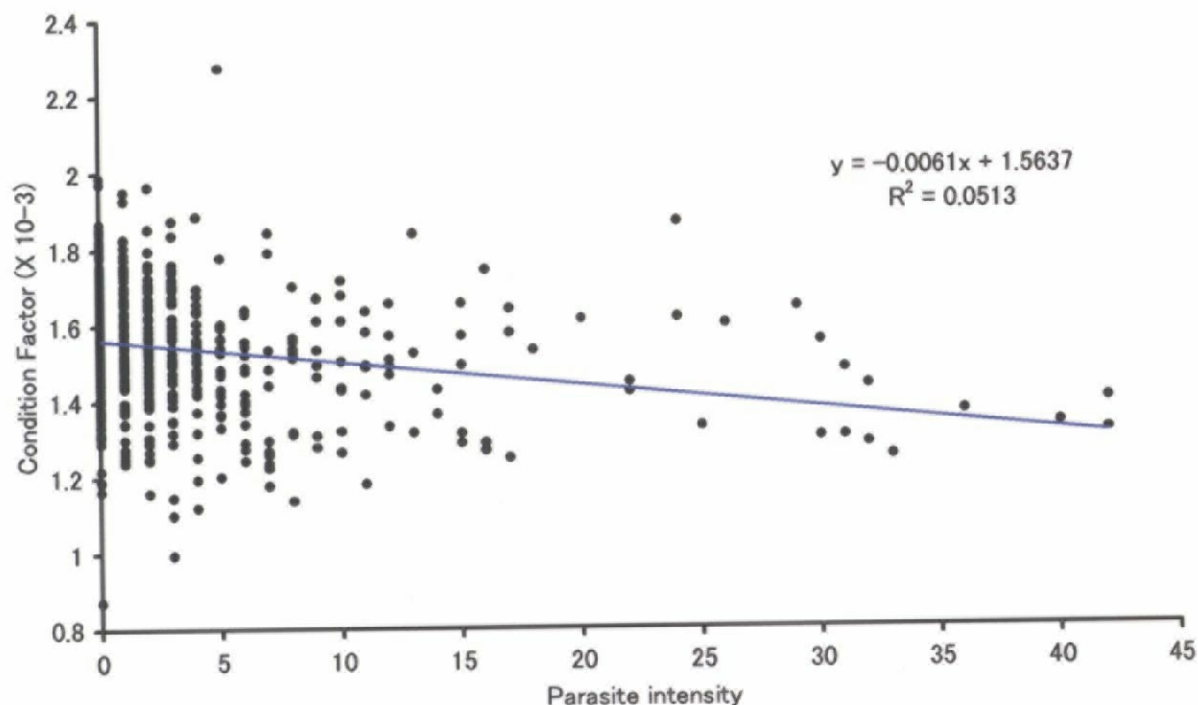


Figure II-11. Relationship between intensity of *N. hirame* and host condition factor for 2002yc from Obama bay. Correlation coefficient and the equation of best fitted line is shown on the graph.

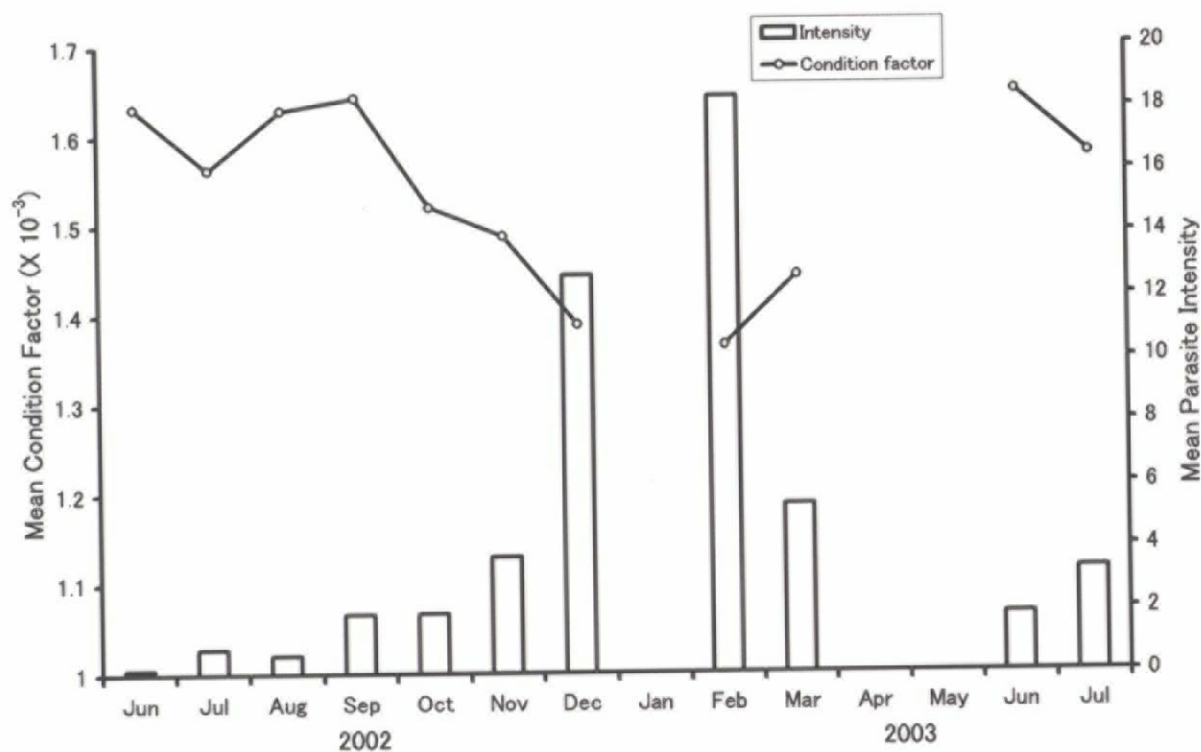


Figure II-12. Change in condition factor of flounder and parasite intensity for 2002yc from Obama Bay. Monthly samples of less than 10 was excluded from the graph.

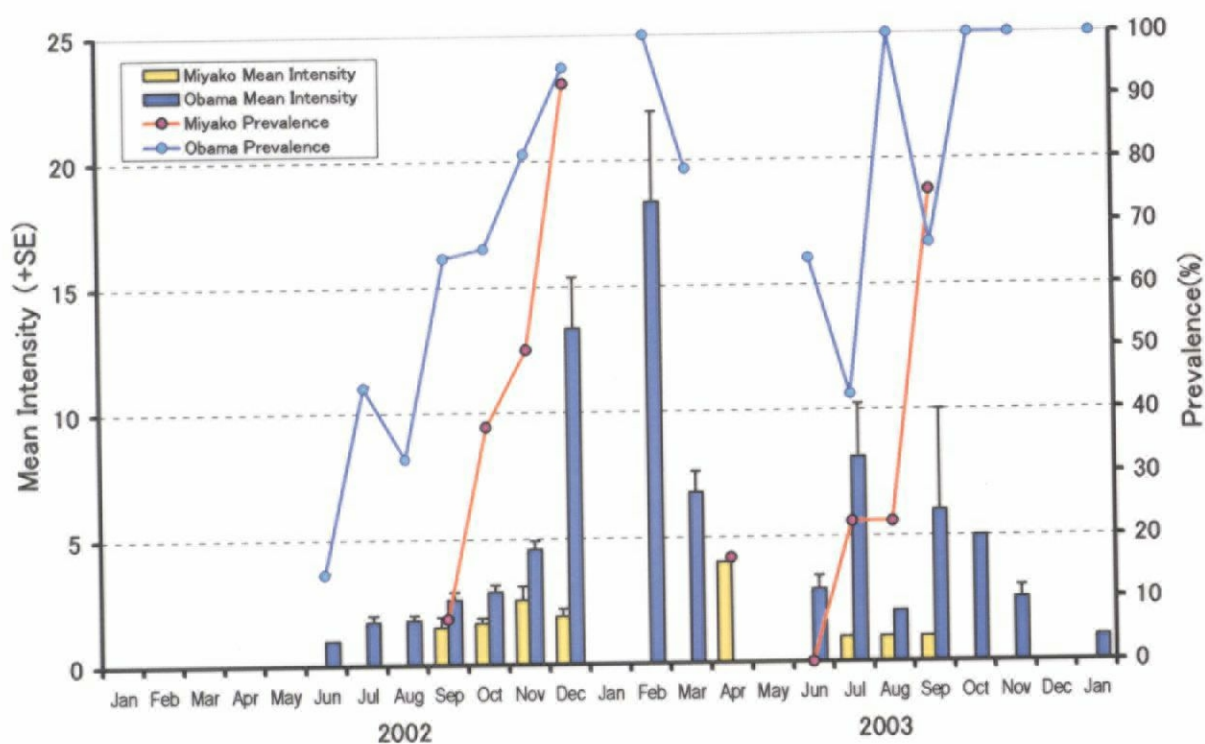
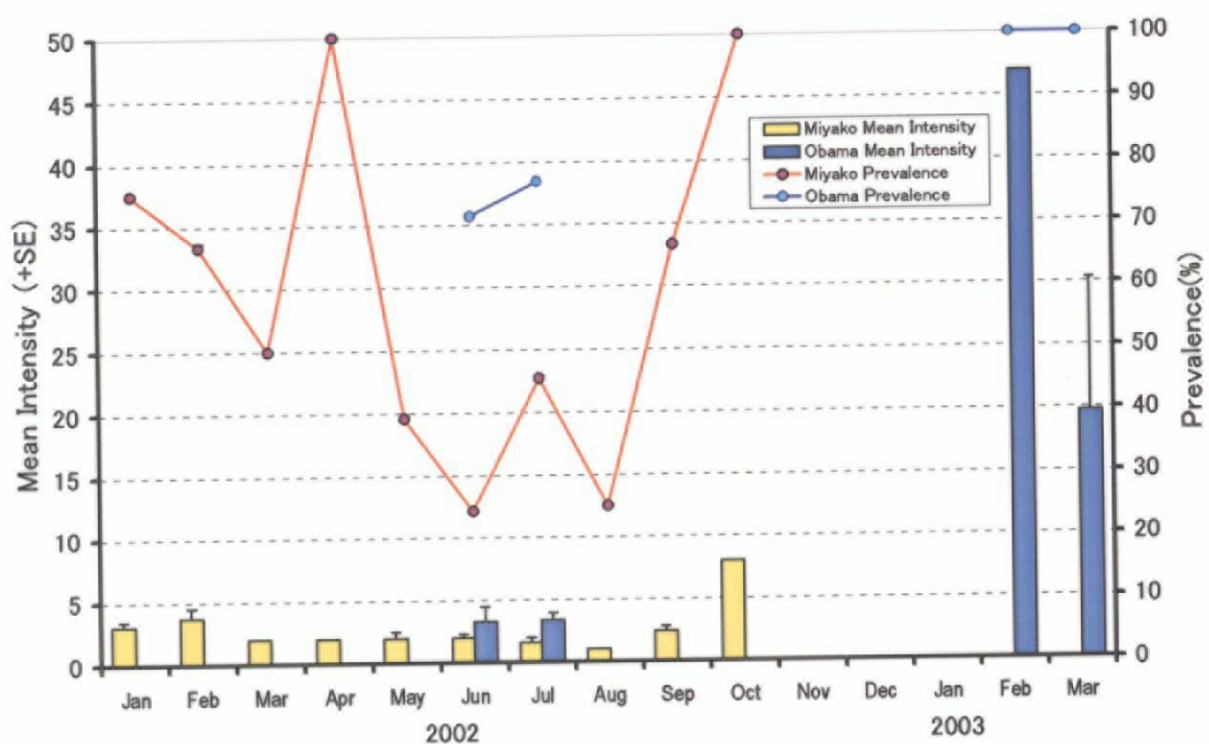


Figure II-13. The mean intensity (bar) and prevalence (line) of the fish captured in Miyako Bay (orange) and Obama Bay (blue). Year class 2001 (top), 2002 (bottom) 2003 (next page) were plotted separately.

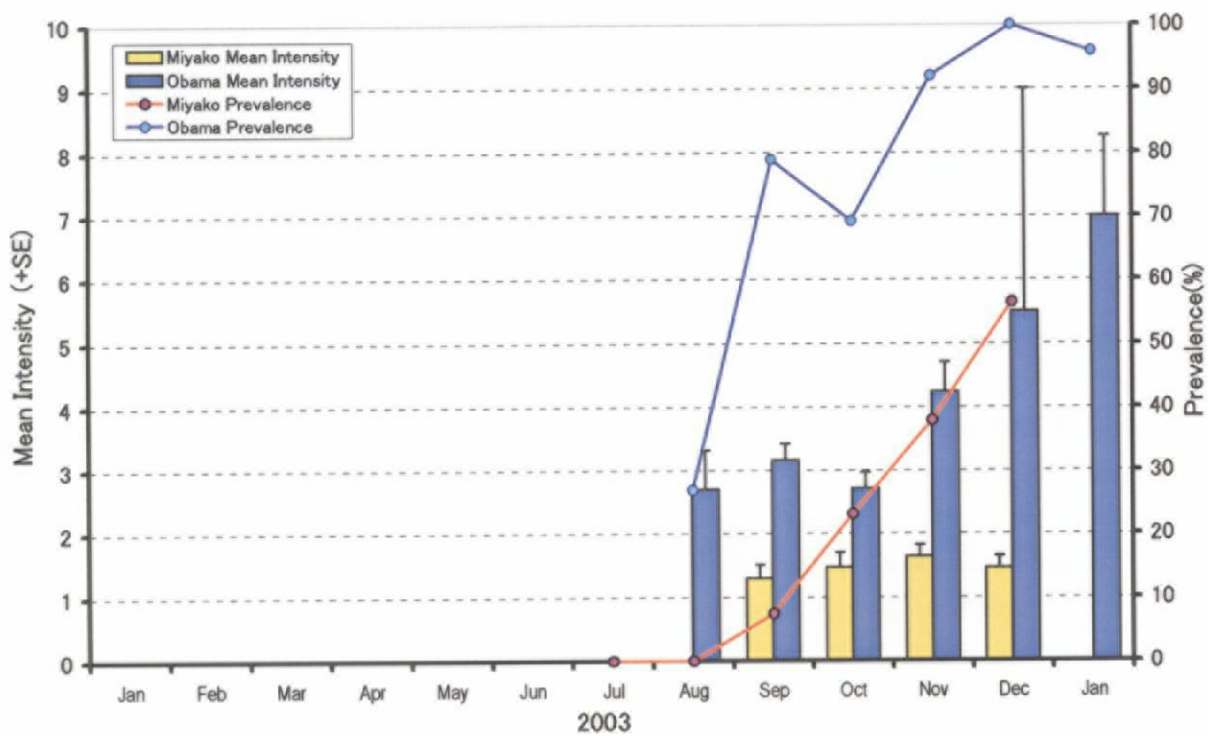


Figure II-13. Continued

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